

Is Britain a part of Europe?

The British decision to reconsider membership of CERN suggests backsliding from Europe. But it is only another sign that the administration of British science should be improved.

EVER since the British occupation of large parts of what is now France ended in the fifteenth century, the British have been only ambivalently linked with mainland Europe. Last week underscored that well-known truth. The predictable (see *Nature* 15 March, p.213) but nearly-averted collapse of the European summit has put the British Government once more in the European dog-house, with the justice of its case inadequately understood. The British decision that there should now be a reconsideration of continued British membership of CERN, the European collaboration in high-energy physics, will further reinforce the impression that the British have concluded that their sceptered isle north of the white cliffs of Dover would be better off on its own. Although membership of the Community and of CERN are far from identical, and although the implications of British membership of the Community are by far the greater, the common elements in last week's events are chilling.

On both issues, the British have a reasonable case. Within the Community, the importance attached to agriculture is anomalous, deriving from the 1950s. To belong to an organization whose budget cannot be controlled is foolish. And there is no longer any reason why only two members of the Community, Britain and West Germany, should be net contributors to the total cost of what has become a genteel way of converting most countries' gross contributions into farm subsidies. The Brussels meeting last week came close to a recognition of these complaints and also had the benefit of a scheme for containing the cost of European agriculture. So why did it fail? The outward reason, for which Mrs Margaret Thatcher, the British Prime Minister, has been blamed, is that the British insisted on the full amount of the monetary compensation they had been demanding (which is not true). The real reason is that, in the end, the negotiations turned on an arithmetical calculation of national advantage in isolation from a necessarily less tangible consideration of the opportunities the European Community provides. If last week's disagreement is not resolved, the British will be culpable not for having fought for their national interest but for allowing that to be too narrowly defined.

Uncertainty

The danger in the British reconsideration of membership of CERN is that the end result may be the same. Again the case is reasonable enough. Faced with an implacable government, the same that negotiated in Brussels, the Advisory Board for the Research Councils has concluded that it may have drastically to draw in its horns (see p.390). Considering withdrawal from CERN seems reasonable enough. But neither the board nor the Science and Engineering Research Council, which administers the budget for high-energy physics, has felt qualified to take a view off its own bat. Instead, a difficult decision has been farmed out to yet another committee and what is bound to be a delicate diplomatic issue has been transformed into a simple exercise in Boolean algebra: the review committee will make a YES/NO recommendation, the board will give a YES/NO endorsement and the government will respond YES/NO. For the best part of the two years, CERN will not know whether Britain is going to remain a member, but the financial pressure on the research councils will not be relieved until 1987, by which time it will be too late, on the board's own protestations, to rescue British science.

What else could have been done? The starting point should have been that diplomatically delicate issues must be dealt with diplomatically. Although the British position differs from that of most other CERN members in that the annual subscription competes directly with the funds generally available for supporting research, the British interest to pay less is probably widely shared. Why not find out? But even if there is a common interest in a cheaper CERN, that would not imply a mass exodus. There has never been an occasion when the cost of operating the laboratory has been examined by people who are also sympathetic to its objectives and conscious of its achievements. Why not mount that for starters? Indeed, why not have done so two or three years ago, when it became clear that CERN membership would be a problem? And since the objective is not to abandon high-energy physics as such but to make room in a stagnant British science budget for other things, why not give warning well in advance that the next large accelerator to be started should be built by an even wider collaboration? None of this would have saved money in the short run, but nor will the present plan. But the result would have been a better understanding of the problem, perhaps a measure of economy and a decision on future membership reached in concert with others, not one presented by Britain to the rest of Europe as the last word on the national self-interest.

Diplomacy

But how could this have been accomplished by a board whose function is merely advisory? No way. The board as constituted cannot engage in diplomacy or instruct civil servants to do so on its behalf. For these are tasks that either the government or the research councils alone can undertake, while the advisory board has no employees of its own, only people told off by civil servants to help it out. And because nobody is sure whether it is an independent body giving independent advice or, alternatively, a kind of representative body intended to conjure consensus from the necessarily different interests of the agencies its members represent, it is bound to be even less capable of making up its collective mind than most committees. This is not a complaint against the members individually, but at the conditions under which they are brought together. Ordinarily, these difficulties are not conspicuous. The danger, illustrated by the way in which the decision about continued membership of CERN has been forced on ABRC and then shelved, is the impression thus created that Britain is once again dealing high-handedly with the rest of Europe. It is hard to think that any single member of the committees concerned would have allowed that to happen.

Things can only get worse in the months ahead. The board, having committed itself to the belief that the British Government should spend more on basic science, has no choice but to reiterate that view with each successive piece of advice it gives. Its decision to commission a study of trends among comparable countries may help to make its repeated complaints seem reasonable, not merely strident. But the board does not have the power to do what might most effectively help to make its case — to enquire into the way in which its dependants manage their affairs in such detail as to demonstrate the good sense of efficiency of what is being done under its wing. The complaints of one of the board's newest members, Sir Douglas Hague (see p.391) could hardly have been better timed. □



How to keep secrets

The United States should be more flexible about the flow of militarily sensitive technology.

THE Reagan Administration is making a muddle of its revision of the Export Administration Act, the measure that will regulate the export of technology with military implications. Long before the original version was due to expire last September (it has been extended), the administration was openly complaining about the rules it had inherited. It is now more than two years since Admiral Bobby Inman, then still with the Pentagon, startled academics with the statement that the flow of militarily important information from the United States to the Soviet Union constituted a haemorrhage and that if universities did not volunteer a mechanism for abating the flow, they would find one thrust upon them. That is what is now happening, to judge from the threat by several research universities that they will accept no more research contracts from the Pentagon if foolish restrictions on publication are enforced (see opposite). Most people's instinctive reaction will be to blame the Pentagon for its failure to understand the character of academic science. Blame attaches, but the issues are too complicated for the blame to be unalloyed.

The need for legislation of some kind is regrettable but not disputed, except by the Soviet Union. National defence has become so bound up with technology that all governments seek to keep potentially valuable secrets to themselves. The Soviet Union has the simplest and most effective arrangement there could be. Military research and development is kept strictly separate from civil research and development, with the consequence that the technical community is split into two and that the pace of civil development is slower than it would otherwise be. Such a rigid division between military and civil work would be intolerable in most Western countries for two reasons: the rigidity of the Soviet system is an offence against people's liberty, but the restraint of civil development is both unnecessary and intolerable. So much is widely understood. It is less widely appreciated that shading of the separation between military and civil research and development can be purchased only at a price — leakage of information and even products.

Tension

This principle is implicit in the arrangements that have grown up in recent decades in the West for regulating the flow of technology to the East. There is a NATO-based organization called COCOM that maintains a list of products in which trade shall not be free. Governments which honour the embargo need domestic legislation allowing them to determine how their own companies should conduct their export businesses, which is partly what the Export Administration Act is for. What the US Congress needs to appreciate is that the system for operating the strategic embargo has never worked smoothly, and never will. In the strictly commercial field, where what matters is the export of products, there are several sources of tension between the United States and its partners. First, perceptions differ on the urgency of the strictly military threat. Second, there is a continuing dispute on the degree to which strategic embargoes should be devised for economic rather than military reasons. Third, many companies and governments in Western Europe regard Eastern Europe and the Soviet Union as the principal markets for technologically advanced products which are accessible to them. Finally, there are suspicions that the strategic embargo can be used by the United States as a means of putting other Western companies and governments at a disadvantage, especially in the sale of goods made in Western Europe under licence from the United States.

Congress has entered into the revision of the act with the gusto expected in an election year. The result is that the Senate and the House of Representatives have emerged with bills so laden with contradictory amendments that it is hard to see how they can be reconciled. On some issues, the administration has been helpful, and on the side of the angels. Now, for example, the suspension of all trade with the United States will not be the automatic penalty

for a Western European government judged (in Washington) to have administered the strategic embargo loosely. But in a year when there is some electoral benefit to be won by those who let their constituents know clearly where they stand in relation to countries overseas, the temptation to burden the bill with tough-talking amendments has been irresistible. Both the administration and Congress must, however, share responsibility for the most obvious weakness of what they are planning, their failure openly to recognize that in the West, no strategic embargo can aim at complete prohibition. The best that can be done is to keep the flow of goods and information at such a level that the natural pace of development in the West will always outdistance the advantages gained from leakiness.

Computers

How is such a principle to be applied to information as well as to the export of goods and services? Hitherto, the COCOM mechanism has at least been a forum in which contentious issues can be resolved between the United States and its partners in the enterprise. What has worried Western Europe in the past few years has been the tendency in the United States to a rigid interpretation of technological advantage. Computer equipment naturally features on the embargo list for example, and there would undoubtedly be great strategic significance in, say, the export of machines for making microprocessors, but in this respect the interests of the military and commercial secrecy luckily coincide. On the other hand, there is very little of strategic value to be learned from a few examples of hardware put on the markets in the West five or so years ago that cannot just as easily be learned from the open literature. Much the same is true of another fashionable field, the manufacture of novel materials. The fact that, with the passage of time, many innovations in such fields are found to have been used in the manufacture of Soviet equipment is neither here nor there. All that should matter is that the time-lag should be long enough to ensure that techniques in the West have moved on another notch.

This is the point at which the US academic community has something to offer the administration. In the past few months, with the growth in the number of supposedly public conferences which have been restricted to US nationals, it has become plain that too many decisions about the administration of the whole system have been left to officials who are plainly ignorant of the realities of technological innovation, and of the comparative ease with which innovations once launched may be imitated elsewhere. Is it too much to ask, even at this late stage, that the administration should engage the academic community in a serious attempt to define the effectiveness of policies such as those now being followed? Some case histories would help, as would a serious study of the two-way flow of information between the United States and Japan on the development of microelectronic devices. (The officials responsible for excluding non-US nationals from conferences in the United States seem unaware of the numbers of Japanese names with which conference programmes of the IEEE are now littered.) And it should be possible to make some qualitative assessment of the degree to which the inhibition of the free flow of information may damage technological innovation even in the United States.

At some level, however, that is the coinage in which the price of secrecy must be paid. Reports that some research universities in the United States will not accept research contracts if normal publication of results is seriously inhibited are merely a proof of that. For many universities in the United States and their faculties, this is an issue that cannot be fudged. Senator Jesse Helms' amendment to the Export Administration Act is bound, if it stands, to force such a confrontation. But there is no reason to think that the universities in this dilemma are disloyal to the administration, or anxious to see some other power prosper. The essential difficulty is that the definition of what information is sensitive cannot be left by self-respecting academics to nameless officials in some government office. Here again the need is for a forum for framing definitions that command the respect of those affected. □

Research freedom

US academics jib at Pentagon secrecy

Washington

TWO months after unveiling the biggest research and development budget in its history, the US Department of Defense (DoD) is facing a major crisis in relations with the academic research community. The presidents of several leading research universities, believed to include Harvard, Stanford and Massachusetts Institute of Technology, have drafted a letter warning DoD that they may refuse in future to accept "sensitive" applied research contracts from the department if it implements a draft directive limiting the right of university researchers to publish.

The controversial directive, DODD 2040.2, was the main item discussed at a meeting last week of a joint university-DoD working group looking into the impact on universities of the Reagan Administration's crackdown on the transfer of sensitive technologies to potential military adversaries. The meeting ended without agreement after the university side warned that some of the nation's best universities would withdraw from defence research altogether rather than sign contracts giving DoD the right to decide whether or not research findings should be published.

Under the directive, DoD would have no right to restrict publication of papers which emerge from contracts in research areas designated in the contract as "non-sensitive". Nor would it block publication of "sensitive" basic research papers, although it would have a chance to see them before they were submitted and ask authors to consider making changes or withholding publication. The universities' main complaint concerns papers which are both "sensitive" and "applied". In these cases, authors would have to give the papers to the Pentagon 90 days before submission to a publisher. And DoD would be allowed to insist on changes or to block publication.

DoD is already empowered to classify as secret any research findings which, if released, could seriously jeopardize the security of the United States. At last week's meeting, Gerald Lieberman, Stanford University vice-provost, said he would prefer DoD to use the formal classification procedures in such cases than to ask universities to surrender control over a wide range of research contracts. And David Wilson, the University of California political scientist chairing the university group, warned that a number of universities (he would not name them) had already signed a document saying they would refuse to accept the blanket control of "sensitive" applied projects recommended in the directive.

The universities calculate that formal classification will be used rarely, because of the strict requirements government departments must meet before proving the need for secrecy. DoD, arguing that the proposed new measures will also be saved only for rare occasions, believes it would be a pity to adopt an "all-or-nothing" approach based on classification. The two sides are to meet again to look for a compromise.

Meanwhile, the universities are facing an unexpected setback on another front in the battle against intrusive controls on research publication and dissemination. In May, pressure from the universities led to significant changes in new drafts of the expiring Export Administration Act — one of the major pieces of legislation used by

the administration to staunch the flow of militarily useful information to the Soviet Union. Committees in both the Senate and the House of Representatives added language to the act asserting that it was the policy of the United States to sustain a "vigorous" scientific enterprise. "To do so," the declaration added, "requires protecting the ability of scientists and other scholars to communicate their research findings by means of publication, teaching, conferences and other means of scholarly exchange". The House of Representatives has adopted the new language, but the Senate, in voting for a package of amendments proposed by North Carolina Republican Jesse Helms, has added the word "non-sensitive" in front of the phrase "research findings". The final wording will be decided by a House-Senate conference, and in preliminary staff meetings last week there was no indication that either chamber was prepared to modify its language. The Universities, however, take comfort from the fact that Senator Helms is not a member of the conference committee.

Peter David

Urban research

Cambridge fights nerve gases

Boston

THE city council of Cambridge, Massachusetts, is embroiled in another scrap with a local research institution. Three weeks ago, the city's health commissioner ordered Arthur D. Little Inc., a prominent consulting company, to cease research on toxic nerve gas and blister agents in its new high-level containment facility until an advisory committee appointed by the city manager had approved the work. A.D. Little responded by obtaining a preliminary injunction against the order of the county court two weeks ago, and the judge will rule on the company's request for a permanent injunction this week.

The controversy over the new Phillip L. Levins Laboratory began after its dedication last fall, when it was widely reported that A.D. Little had contracts from the Department of Defense to investigate ways of detecting, detoxifying and destroying highly toxic "surety agents" — chemicals whose primary use is killing people. Despite the company's protests that the containment and safety precautions far exceeded federal requirements and that the amounts of the agents to be tested were quite small, Cambridge residents living nearby were jittery.

At a tumultuous meeting in November, the city council voted to appoint a scientific advisory committee to review the proposed research. They had made little progress by February, when the Massachusetts Department of Health approved the nerve gas work, finding that the laboratory posed minimal risk to the surrounding community. Ironically, this announcement revived opposition, particularly in the

neighbouring cities of Arlington and Belmont. Suspicious of the reports approving the laboratory and preferring to find out for itself, Cambridge City Council quickly requested a ninety day moratorium on work at the facility, to which the company refused to agree, citing its contractual obligations. Considerable pressure was then put on the city health commissioner, Melvin Claflin, to use his power to ban the work as a danger to public health. Despite his opinion that the laboratory was a safe facility, he issued the order prohibiting the testing, storage, transportation or disposal of nerve gas and blister agents within the city.

Left with only the option of destroying material on hand, A.D. Little argued in court that such an action would violate federal statutes that outlaw the destruction of US property, the destruction of chemical surety agents, without the permission of the Department of Defense. At the council meeting three days later, tempers flared as A.D. Little's president John Magee was denounced by a frustrated Councillor Alfred Vellucci, best known as an initiator of a ban on recombinant DNA work in Cambridge from 1975 to 1977.

This controversy could drag on in the courts for some time. Yet legal resolution of this particular issue will do little to allay public concern about the huge variety of hazardous materials in use at local universities, hospitals and corporations. Many Cambridge residents have expressed wonder at why such work — no matter how minimal the chance of a major accident — has to be carried out in such a densely populated area.

Christopher Earl

European collaboration

UK to brood on CERN pullout

THE United Kingdom continues to pull up the drawbridge to Europe. The British research councils are to spend the next year making up their minds whether to pull out of the European Organization for Nuclear Research at Geneva (CERN). The decision was announced last week, on the heels of the demonstration by Mrs Margaret Thatcher, the British Prime Minister, at Brussels of her independence from Europe, by Sir David Phillips, chairman of the Advisory Board for the Research Councils (ABRC).

ABRC says that the need to review membership of CERN has been made necessary by the "grave" shortage of funds for the support of civil research in Britain. Two requests for extra funds in the British research budget have been turned down by the government.

The politically sensitive nature of the planned study seems in everybody's mind. The research councils last week



Mrs Margaret Thatcher gets to know CERN.

emphasized that the British commitment to CERN is a treaty obligation which can be ended only by the government. In a letter to the ABRC chairman, also released last week, Sir Keith Joseph, the Secretary of State for Education and Science, insists on the "constitutional distinction" between the "board's advice on a matter and what the government may decide on that advice".

The ABRC plan is that a committee under Sir John Kendrew, president of St John's College, Oxford, president of the International Council for Scientific Unions, chairman of the British National Scientific Committee on Unesco and, until 1982, director-general of the European Molecular Biology Laboratory, will report both to the Science and Engineering Research Council (SERC), which launders the British contribution to CERN, and to ABRC.

The other members of the committee are Sir Jack Lewis (physical chemistry, Cambridge), Professor Kenneth Pounds (X-ray astronomy, Leicester), Sir Francis Tombs (previously with the UK Atomic Energy Authority, now a director of manufacturing and banking companies) and Sir Douglas Hague (chairman of the Economic and Social Research Council). Dr Christopher Llewellyn Smith, the high-energy physics theoretician from Oxford with close links to CERN, is to be an adviser to the review group.

The group, to which other members may be appointed, is expected to report a year from now. On that timetable, given the need for a full calendar year's notice, British membership could not end sooner than 31 December 1986.

The research councils seem to have won from the government the concession that if it is eventually decided to withdraw from CERN, the money saved will be "redeployed" within the science budget. Or, in Sir Keith Joseph's words, "While the level of the science budget for any future year will, of course, need to be considered in the light of all the circumstances at the time, I can say definitely that any such proposal to which the government agreed would not be

an occasion for cutting the science budget as a whole".

On the question of what would happen if the British Government decided for political reasons not to follow an ABRC recommendation to withdraw, Sir David Phillips said that he would expect the government to "stand by its commitment".

But Professor John Kingman, chairman of SERC, denied that the proposed study of withdrawal from CERN is a stratagem to saddle the government with separate financial responsibility, saying that no part of his research council's programme is "sacrosanct", that another committee is at work on commitments in space research and astronomy and that the research council contribution to CERN is the largest identifiable item in SERC expenditure — some 24 per cent of the total.

There was some confusion last week about the possible abruptness of British withdrawal from CERN. In one breath, Kingman said that "you cannot be an 80 per cent member" and offered the possibility that "our partners would agree to a different pace of development". The review group's terms of reference ask it to consider "the role and extent of international collaboration", suggesting the possibility of renegotiation of the treaty, not outright withdrawal.

By 1987, the first possible year in which the United Kingdom might not belong to

Another blow to UK physicists

BRITISH nuclear and high-energy physics are under pressure other than that of the threat of withdrawal from CERN. At last week's meeting of the Science and Engineering Research Council, when preliminary plans were drawn up for spending during 1985–86, nuclear and high-energy physics were at the bottom of the list.

The council appears to be engaged on its "forward look" for submission to the Department of Education and Science before the budget cycle for the next financial year gets under way. The trick on which the council has embarked, with the objective of introducing flexibility into its budget, is to induce its component spending boards to release a portion of their funds into a general reserve on which all may then make bids. On the showing so far, the science board (small science) seems to be doing best, then engineering and then space science. Increases in these fields will then be paid for by a decrease in the budget of the nuclear science board.

Broadly the argument seems to have gone this way: small science has been relatively under-supported and so should receive more from the general reserve. Engineering, still favoured by the government, has risen threefold in the past decade so that the council leant towards consolidation. But space science has been badly hit both by the policy emphasizing optical astronomy and the general shortage of

funds and so should also be protected.

The upshot is that there was nothing in the kitty for the nuclear science board, which will be left with an even smaller margin than the present £15 million between its total expenditure and the treaty-determined subscription to CERN. Some high-energy physicists say that the margin is already so eroded that the CERN subscription is ill-used by the British high-energy physics community. These proposals, to be finalized in April, will become part of ABRC's proposals to the British Government, due in the spring. A more radical proposal for internal reorganization is a plan to merge the two most expensive boards maintained by SERC — the astronomy, space and radio board and the nuclear physics board. This proposal, first canvassed by SERC chairman Professor John Kingman last summer, is intended to allow peer assessment of the relative merits of high-energy physics and astronomy projects. At present, decisions such as these fall to the council, which does not always have the scientific competence to make judgements.

The effect of such a decision would be to create a single high-spending board accounting for some 40 per cent of SERC expenditure. An obvious snag is that the big spenders and the small spenders would be set at each others' throats. But council meetings might be shorter. Robert Walgate

CERN, the LEP electron accelerator will be nearly complete and plans for the next more powerful machine would, on past form, have been canvassed. (LEP is due to be commissioned during 1988.) So, at the least, the mounting of the review is probably a guide to future negotiating positions at CERN. But British participation in planned experiments at LEP would entail some financial contribution in 1977 and thereafter.

The decision to reconsider British membership in CERN has sprung from ABRC's annual recommendation to the British Government on the division of the

science budget among the several claimants. This year's document, published three months late because of the delay in mounting the CERN inquiry, says that it has been forced to consider "withdrawing completely from a major area of scientific activity" because the problems facing the research councils are "so grave".

For an occasion of such solemnity, last week's announcement was carried off light-heartedly, even with jocular. The explanation may be that which has people giggling at funerals, perhaps merely that the announcement was made to a bunch of journalists.

John Maddox

UK research budget

Straitjackets for all

THE British Advisory Board for the Research Councils (ABRC) is likely for the third successive year to urge on the government that more money should be spent on civil research, according to its chairman Sir David Phillips. He was introducing last week an account of the board's frustrating dealings with the government over the research budget for the financial year beginning next month.

In a break with practice, ABRC last year gave the UK Government two substantial pieces of advice — a statements of its case for more money (in time for the beginning of the annual estimate of public expenditure in the spring) and recommendations on the distribution of the budget eventually agreed (in the autumn). Both documents together with correspondence with the Secretary of State for Education and Science are published as *Scientific Opportunities and the Science Budget 1983* (available from Publications Despatch Centre, Department of Education and Science, Cannons Park, Stanmore, Middlesex).

ABRC's bid for 1984-85 turns out to have been for an extra £22 million, or some 4 per cent of the total science budget. In the two succeeding years, ABRC asked for £33 million and £43 million extra, making a total for three years of £98 million after allowing for inflation (see *Nature* 15 March, p.217).

ABRC would have spent the extra as follows:

Research grants. The documents note that the three principal grant-making councils rejected 650 "first-rate" applications in 1980-81, for lack of funds. Making good the shortfall would have required £3.8 million in the coming financial year and £7.6 million in 1986-87.

Manufacturing engineering. The Science and Engineering Research Council (SERC) wanted to spend £4 million in the coming year, increasing by £1 million in each of the two succeeding years, on the application of computer techniques to manufacturing engineering.

Food and nutrition. An expanded programme in food research and nutrition by

the Agricultural and Food Research Council (AFRC) would have cost £18.5 million over three years, but £11.5 million of this would have been found internally.

X-ray astronomy. A plan by SERC to launch six years from now an X-ray satellite capable of detailed spectroscopic measurements would have cost £12.5 million over three years and £30 million in total. ABRC says that SERC has now decided not to proceed with this plan.

Medical projects. The Medical Research Council (MRC) had a shopping list of projects in neuroscience, nutrition and diagnostic imaging costing £12.5 million over three years.

Biotechnology. AFRC sought an extra £6 million over three years, chiefly for work in plant science.

Geophysics. The Natural Environment Research Council (NERC) sought to spend a total of £12 million over three years on projects such as sea-surface topography by satellite imaging, remote sensing and deep

seismic surveys.

In addition, ABRC sought £22.5 million over three years to meet the costs of "restructuring", chiefly at AFRC and NERC, which will now be met by a "tax" on the budgets of MRC and SERC in the last two of the next three financial years.

By the use of words such as "regret", ABRC has left the government in no doubt of its discontent that extra funds are not forthcoming. It says that growing demands in the past few years have overstretched the research councils and that even the greater flexibility likely to flow from "restructuring" (chiefly reductions of in-house commitments) will not allow the councils to exploit all the opportunities which arise. "This requires a continuing investment", which can be achieved only by a larger budget or by "withdrawing completely from expensive scientific projects". ABRC asks for two further assurances from the British Government — a lasting understanding with the government on the effects of sterling devaluation on the cost of international subscriptions and on the effects on the research councils of the decline in research commissions from government.

The tactics of ABRC's dealings with the British Government from now on are obviously delicate. Professor John Kingman, SERC chairman, said last week that the research councils hope to persuade ABRC to keep on complaining about the shortage of funds. But too much stridency may simply irritate the government.

It may be significant that ABRC has for the first time set aside £50,000 of the total science budget for "science policy studies", the first of which is to be an attempt at an international comparison of spending on basic science in Britain with that in the United States, West Germany, France and the Netherlands. □

Hague raises doubts over ABRC

SEARCHING questions about the constitution of the Advisory Board for the Research Councils (ABRC) were raised by one of its newest members, Professor Sir Douglas Hague, chairman of the Economic and Social Research Council in the Mond Lecture at the University of Manchester on 12 March. His general complaint seems to have been that ABRC is unable, because of its constitution, to manage its affairs.

Sir Douglas acknowledged the complexity of the range of problems with which ABRC is required to deal but remarked on the overlap between its field and that covered by the Advisory Council for Applied Research and Development (ACARD), asking whether the division is tenable or useful. He also raised the question whether a body whose function it is to recommend a division of the science budget between the research councils should as a matter of principle include the heads of the research councils as members.

He said that "ABRC needs to change... and its bureaucracy needs to change". He argued that there should be some means by which ABRC, the research councils and the various parts of the government bureaucracy involved with them could be enabled to learn from their experience.

Specifically he urged that there should be a "strategy unit" within ABRC to "promote learning" and to ensure that "change actually happens". Acknowledging that think-tanks are out of fashion in Britain, he nevertheless felt that ABRC is in need of strategic advice.

Sir Douglas also dissented from Professor Ronald Mason's recommendation at the end of last year that ABRC should be "strengthened" on the grounds that such a committee would be so powerful that it would be regarded as "omniscient" but would be no better able than the present committee to take initiatives in the development of new fields of research. □

Information technology

Germany increases investment

DRASTIC steps to strengthen West German information technology are outlined in a document now published by its Ministry of Research and Technology (BMFT). The measures proposed are a compromise, intended to counter the failure of West Germany to hold its own against Japanese and US competition, between the Minister of Research and Technology, Dr Heinz Riesenhuber, and the monetarist Minister of Economics, Dr Otto Graf Lambsdorff.

Graf Lambsdorff, who, while at the Organisation of Economic Cooperation and Development (OECD), criticized industrial targeting as a subtle form of protectionism, opposes direct state support for research as a distorting influence on market forces. Dr Riesenhuber, a more traditional pragmatic conservative and a consistent advocate of the government's policy of directing the balance of support away from direct to indirect measures, such as tax concessions, intends, nevertheless to channel DM 3,000 million (£750 million) into information technology over the next five years.

The new BMFT report stresses the crucial role of the European Economic Community (EEC) in creating a more homogeneous rationalized market for European companies, now hampered by the small size of their national markets and the mutual incompatibility of their pro-

ducts. The report asserts that liberalization of European public purchasing policy in fields such as education, science, telecommunication and defence may be more important even than the collaborative Esprit programme (*Nature* 309, 100; 1984). A key question is the normalization of standards (see below) and whether or not this will make European products compatible with IBM. A recent article in the weekly *Die Zeit* highlights the difficulty of a major German institution, the Dresdner Bank, in making the investment decision involved in the choice between IBM and the major non-compatible contender, Siemens.

West Germany will begin work at home. The government intends to adopt a more creative domestic acquisitions policy and will insist on conformity with official norms for products and interfaces and the free flow of information needed to ensure compatibility.

The West German post office is to embark on five-year and ten-year programmes for the digitalization of telecommunications and the introduction of optoelectronic communications. Integrated Services Digital Network (ISDN) will be tested in 1985, introduced from 1987 and should be complete in ten years. The introduction of a broad-band ISDN will begin in 1989. Optic communication

systems are already being tried out and the results of the trials are expected in 1985-87. BMFT estimates that there will be 1 million West German subscribers to the European mobile radio telephone system. One tender for this system, presented this week by Standard Elektrik Lorenz of Stuttgart, AEG-Telefunken, and SA de Telecommunication (SAR), Paris, claims to offer a revolutionary new digital systems that could open a much wider market.

BMFT itself is to support a research communications network linking academic and research institutes, while the Deutsche Forschungsgemeinschaft will increase support for basic research in this field by DM 100 million.

Specific provisions for research and development from BMFT's five-year budget include DM 90 million for interfaces, DM 90 million for optoelectronics, DM 200 million for components, and DM 530 million for industrial automation. Applied research will be mainly by the Heinrich Hertz Institute and the Society of Mathematics and Data Processing of the Fraunhofer Gesellschaft, which will put about one quarter of its budget into microelectronics. It will coordinate microelectronics within its 30 institutes and with the business, academic, institutional and *Länder* regional communities.

Meanwhile Siemens, with Nixdorf one of the main competitors to IBM in the home microelectronics market and the only European producer of 64-kilobit microchips, last week announced that it will be taking on 100 new employees at its development and pilot plant at Perlach, Munich, where work will begin this year on a 256-kilobit chip. It plans to invest DM 600 million in a 256-kilobit chip production plant at Regensburg. Nixdorf, which goes public this year, plans that some DM 500 million of its new capitalization will be spent on research and development. The outcome will not, however, be soon enough to relieve the shortages of microchips caused by economic recovery in West Germany, as elsewhere, and which Siemens considers may last until the second half of 1985. Canny customers have ordered ahead, but deliveries of equipment have been seriously delayed.

The BMFT report notes that the government's pilot project on the founding of small high technology businesses with private capital lays special stress on information technology. Although small microelectronics companies trickle onto the stock exchange, the Christian Democrats-Free Democratic coalition's continued aim is to create international and national frameworks for the faster translation of high technology into industrial wealth.

A Swiss analyst group estimates that the fact that Europe lags behind Japan and the United States in microelectronics costs four million jobs and that three-quarters of all jobs within the EEC are dependent directly or indirectly on microelectronics.

Sarah Tooze

Europe sets a new standard

Brussels

EUROPE's major computer firms have taken a far-reaching step to create a real European market in information. Twelve leading information technology companies have jointly proposed to the European Commission a programme for the implementation from 1985 of standards that should enormously increase the possibilities for intercompatibility of products.

The twelve companies — AEG, Bull, CGE, GEC, ICL, Nixdorf, Olivetti, Philips, Plessey, Siemens, Stet and Thomson — have been the driving force behind the European Commission's Esprit programme.

They have now agreed on an Open System Interconnection (OSI), described by the Commission as a "standard for standardizers". Basically defined by a CCITT/ISO standard, it will permit easier exchange of information between terminals, computers, people and networks and will allow the maximum interaction between all aspects of computing, from the data processing and process control systems of today through to electronic mail, telex (supertex), and the automated office of tomorrow.

OSI will cover seven "layers". The first relates to the physical layer, whether telephone wire, optical fibre, bus, coaxial

cable or satellite. Higher layers cover data link, network, transport of communications, session, presentation and application functions.

The twelve information technology companies have proposed that a selection of OSI standards should be implemented from next year with OSI eventually



producing a full catalogue of interworking products. They want to define demonstrator projects which will show the interworking of such products in action. Public procurement, they say, should be based on these standards as this represents a critical market for its success.

For its part, the Commission is studying the practical aspects and intends to consult governments, national authorities, industry and other users.

David Price

UK microelectronics

More public support in prospect

THE British Government is to spend £129 million over the next six years in support for its home microelectronic component production industry. A new extension to the Microelectronics Industry Support Programme, MISP 2, more than doubles the finance previously available, which is now fully committed. The government's action is intended to spur industrial companies to invest £1,000 million in the development and production of integrated circuits during the rest of the decade.

Mr Kenneth Baker, Minister for Information Technology, said last week that Britain has now caught up with West Germany as the largest consumer of microchips in Western Europe. The increased consumption is ascribed in part to the success of the government's separate programme to encourage the use of microelectronics in industry. But now there is concern that Britain should not become reliant on overseas sources for specialized "custom" chips, and the new scheme is aimed especially at encouraging companies to invest in facilities for their development and production.

MISP 1 was worth £55 million and will

have stimulated investment worth £270 million. Its successor is designed to ensure that the growth of British production at least equals the world average. Home production of integrated circuits will be worth £300 million in 1985, roughly a five-fold increase in real terms since 1978.

The new scheme recognizes the extremely high initial capital investment needed for semiconductor production. Grants of up to 25 per cent will be available for both high and low volume production projects and purchase of computer-aided design facilities. Grants of up to £3,000 will be made for feasibility studies.

There may also be some support for development projects if these do not overlap with the government-supported Alvey programme of research and development in information technology. Companies involved only indirectly in semiconductor production will be eligible for support for projects that are likely to improve the availability of materials, and a large part of the new programme will be spent in companies producing semiconductor manufacturing equipment.

Tim Beardsley

Japanese space research

Shoestring programme prospers

Tokyo

THE Japanese Government has given tentative approval to plans for scientific missions to the "Moon and planets" and, at a much later date, for exploration of Jupiter-type planets and asteroids in the final version of its new 15-year space development programme.

The programme is aimed chiefly at the development of a large rocket for the launch of telecommunication satellites by the National Space Development Agency of Japan (NASDA) (see *Nature* 1 March, p.3) but it also gives significant encouragement to the continued expansion of the Institute of Space and Astronautical Science (ISAS) — a unique academic body with its own satellites, rockets and launch sites.

Although the encouragement to ISAS from the government is most welcome, Professor Minoru Oda, its director-general, is quick to point out that the programme has been deliberately worded in the most ambiguous manner possible. No definite plan has been approved, for there is a great debate within ISAS over which direction to take next.

Despite the apparent illogicality of having separate academic and commercial space programmes, ISAS has so far proved to be a highly successful organization. In the past six years, scientific satellites for the study of the magnetosphere, X-ray sources, solar flares and the middle atmosphere have been launched at the rate

of one a year, along with a series of test satellites, while the annual budget has only just reached 15,000 million yen (\$65 million). The big question now is what is to come after the next three approved missions: an X-ray astronomy satellite (ASTRO-C), an auroral observation satellite (EXOS-D) and Japan's first deep space probes which will fly by Halley's comet (MS-T5 and PLANET-A).

The logical continuation of the PLANET-A mission, given recognition in the government programme, is a flight to the Moon. If the Halley's comet probe is successful, much of the technology for the launch of a lunar probe would be proven. Another strong possibility would be to

continue the successful X-ray astronomy series with the launch of ASTRO-D, a satellite which could complement the United States' planned Advanced X-ray Astronomy Facility (AXAF). Or the line of research begun with the solar flare observation satellite *Hinotori* could be continued with a new mission for the next solar maximum. Again, there is support for launching a satellite to act as a space outpost for the ground-based Very Long Baseline Interferometer (VLBI) now under construction in the United States.

The first decisions are expected in the next few months, for ISAS must soon decide whether to participate in yet another project — the building and launching of one of the four satellites necessary for the US Open Plasma Observation Network.

All these projects — even the launch of a lunar orbiter — are just within the capability of ISAS and its home-developed solid-fuel MU-3S series rockets. But a major question that will have to be answered soon is how long ISAS can keep going with the strange constraint — enforced by the government — that it should develop no rocket with a diameter greater than 1.4 metres. And if it should prove necessary for larger satellites to be launched by the more powerful NASDA rockets, would ISAS then lose some of its closely-guarded independence? **Alun Anderson**

●As well as visiting Japan (*Nature* 15 March, p.216), Mr James Beggs, administrator of the US National Aeronautics and Space Administration (NASA) has been in Europe in search of partners for NASA's manned space station project. Beggs' proposals seemed to be attractive to the Japanese Government — after reassurances that Japanese technology would not be used in any military applications — although it is difficult to see where the money would come from. In Europe there was also a glimmer of hope for NASA. Speaking after the troubled European summit on 20 March, President Mitterrand said that the EEC heads of government had discussed a project that would involve talks with the United States on a manned space station, but added that it was too soon to forecast the outcome. □

Japan expands

Tokyo

JAPAN may soon have a little more territory — if not actual living space — in the Pacific Iwojima island chain, 1,100 km south of Tokyo. A massive under-sea volcanic eruption has been taking place some 150 km north of Iwojima (26° 7' 42" N, 141° 7' 30" E) since at least 8 March — when an aircraft of the Maritime Self-Defense Force (MSDF) spotted the eruption — and now appears ready to form a new volcanic island.

At first, grey and yellow waters were seen pouring from a central spot some 20–70

metres in diameter, discolouring the sea in a plume more than 50 km long and 3 km wide — by far the biggest under-sea disturbance seen in recent times. Flights by MSDF aircraft are now reporting that more than a dozen reefs have broken surface and that rocks are being hurled into the air.

The area is close to the Marianas Trench and some 300 km from the scene of a 1978 eruption that lasted for three months. If a new island forms it will be Japan's fifth in the past 100 years — although only two of them remain. The most recent, Nishi No Shima Shinto, was created in 1973–74 and now has a total area of 200,000 square metres. **Alun Anderson**

Illmensee inquiry

Mixed news on grants

PROFESSOR Karl Illmensee's grant from the Swiss National Science Foundation, suspended last year when doubt was cast on experiments carried out at the University of Geneva, will not be reinstated. Notice to this effect was given to Professor Illmensee in a letter from the foundation dated 14 March. Illmensee said on the telephone earlier this week that he had not yet decided whether to appeal against the decision. "It might be easier to apply for a new grant."

The foundation's decision stems from a meeting of the council of the foundation earlier this month. According to Dr P. Fricker, secretary of the foundation, the council was almost exclusively concerned with the report of the international commission on the Illmensee affair, delivered to the University of Geneva at the end of last month (see *Nature* 23 February, p. 673). Dr Fricker said that the council had considered the report with great care and that its eventual decision to terminate Professor Illmensee's grant was taken unanimously.

Although the commission's report said that it had not uncovered "convincing evidence" of false claims in respect of a series of experiments in the early summer of 1982, the commission also referred to "grave doubts" about some of the work and to "negligent" laboratory practice. The foundation's council seems to have taken the view that such comments outweigh Illmensee's formal exoneration.

Illmensee said earlier this week that the decision was unfair in that it would penalize members of his laboratory other than himself. He said, however, that he was now planning to follow the commission's advice and to repeat the disputed experiments, involving the replacement of nuclei of mouse embryos by those of cells of a teratocarcinoma — ostentatiously undifferentiated cells. Illmensee said that he would be negotiating with the University of Geneva for support for these experiments.

The immediate practical problem seems to be to recover cells of the teratoma, now represented by several ampoules of frozen material. He said that it would take some months to obtain viable cultures and to demonstrate that their characteristics and karyotype had not changed.

According to Illmensee, confirmation of the disputed results should be possible by the end of the year. If the new experiments should confirm those of 1982, Illmensee said, he would invite specialists from elsewhere to visit Geneva to inspect his protocols and verify his conclusions.

Stephen Budiansky adds from Washington: The National Institutes of Health (NIH) are close to a decision on reinstating Illmensee's research grant, which has been held up since May 1983 pending the outcome of the investigation by the University of Geneva commission.

The grant, worth \$74,000, was recommended by the NIH Cancer Advisory Board in January 1983 and was to have run from 1 June. It was suspended in May of that year, when NIH learned of a statement in which Illmensee appeared to acknowledge having falsified data.

According to Mary Meyers of NIH's office of extramural research, NIH are now studying the commission's findings and will decide shortly on the fate of the suspended funds.

Meanwhile, efforts by other researchers to repeat Illmensee's experiments continue to prove unsuccessful. The committee set up last summer to investigate Illmensee's work at the Jackson Laboratory in Bar Harbor, Maine, noted that Illmensee's collaborator there, Dr Peter Hoppe, had tried on his own but failed to reproduce the results he had obtained with Illmensee. These experiments involved removing

nuclei from fertilized mouse eggs to produce single-parent mice and, in other experiments, transplanting nuclei from partially developed embryos into fertilized mouse eggs whose nuclei were removed. In all the experiments, Hoppe prepared the fertilized eggs and reimplanted the embryos, while Illmensee performed the microsurgical removal and emplacement of the nuclei.

Hoppe last week refused to comment on the subject, and would not say whether he is still attempting to repeat the experiments. The Jackson Laboratory committee noted in its report that the "special combination of technical skills" of the Hoppe-Illmensee collaboration could be difficult to duplicate. Hoppe told the committee last summer that he suspected a deteriorated reagent was to blame for his failures. Dorothea Bennett of Sloan-Kettering, who chaired the committee, said last week, however, that she was dubious of that explanation: she said biologists tend to blame "anything" when results cannot be duplicated. □

Soviet Academy

Science to fuel materialism

SOVIET social scientists — particularly the economists and sociologists — are failing to match the achievements of their colleagues in the natural and technical sciences in meeting the demands of the "scientific and technical revolution", according to Academician P.N. Fedoseev, one of the vice-presidents of the Academy of Sciences. Speaking at the annual meeting of the academy, Fedoseev echoed the "sharp and well-deserved" criticism of the social sciences recently voiced by the Communist Party's central committee.

One major problem, for example, which Fedoseev emphasized "cannot be deferred", is the development of a method for calculating the "economic effect" of scientific and technological innovation, and for calculating the effect of economic and social factors on scientific and technical progress. These remarks, outwardly simply the application to the social sciences of Party directives on science in the service of the economy, may nevertheless herald a shift in policy.

Delays in the implementation of research results in industrial and economic practice have bedevilled Soviet planning for years, and the much-publicized "economic effect" of successful innovations selected for medals and awards is calculated, for the most part, on the basis of factory and production-line gross accounting.

Fedoseev's emphasis on the need for a new "methodology" for such calculations suggests that a more sophisticated approach to industrial applications may be on the way. One possibility is that it would take account, for example, of long-term savings due to the greater reliability of a new process, which do not necessarily

become apparent during the first year of accounting. But the unfortunate economists and sociologists could also prove convenient scapegoats for both scientists and industrial managers, when the latter must explain why a new discovery is not yet giving results.

In contrast with the social scientists, representatives of the natural and technical sciences have been singled out for praise. Successes in the food programme range from the indigenous production of plant-protection chemicals to a project for the industrial production of protein from methane and methanol. In the energy programme, a team headed by Academician Anatolii P. Aleksandrov, president of the academy, has completed a detailed breakdown of the Soviet Union's energy needs until the year 2000. Other achievements honoured include the synthesis by genetic engineering of interferon and human insulin, the radar mapping of the surface of Venus and progress of the Kola deep borehole.

But despite those successes, all disciplines seem to have been included in Fedoseev's more general exhortation that scientists should give thought to raising the level of ideological work and to the education and upbringing of the younger generation. The role of science in educating young people in a Marxist materialist outlook is a standard concept in Soviet educational theory, but explicit exhortations to the academy to pay greater attention to the role of science in forming a materialistic outlook and in combating anti-scientific concepts and prejudices strike a note that has not been heard in academy meetings for many years.

Vera Rich

Spanish universities

Tenured posts on offer

Barcelona

LAST year's reforming legislation is now taking effect in Spanish universities and in the Consejo Superior de Investigaciones Científicas (CSIC, the Spanish science research council). In the universities, the process has begun that will allow members of their teaching staff now employed on short-term contracts to become "titular" professors with tenure; and new research positions have been created in CSIC.

The new university selection procedure is a direct consequence of the Law for University Reform (see *Nature* 305, 756; 1983) under which there will be only one way into university teaching. The government intends that the number of professors under contract will be reduced until it represents no more than 20 per cent of the total teaching staff. In some universities, such professors now represent more than 70 per cent of the teaching staff, even though they carry out the same functions as tenured professors. Those now teaching under contract will be free to apply to become titular professors, and this procedure will also be open to Spanish scientists working abroad and to some holders of fellowships.

As many as 8,000 people may be affected by the new law. Only 40–60 per cent of the applicants are expected to be successful; the rest of those now teaching in the universities will have their contracts renewed until 1987, with no possibility of further

renewal. One result of the new procedure will be that most of the teaching staff in Spanish universities will soon be civil servants. Some also fear that there will be very few posts available in the universities for new people, although the government has not gone as far as to accept the view that all the present teaching staff should be given tenure.

The situation in CSIC is quite different. The 56 new posts are not really new, but are vacancies created because people have retired or resigned. Nevertheless, in an institution where the average age of employees is 48, even such a small opening is valuable. When the new presidential team of CSIC took office last year (see *Nature* 304, 202; 1983), it had been hoped that a larger number of new posts would be created. There is likely to be a large number of applicants, some of whom will also be eligible for posts in the universities.

Meanwhile, the Ministry of Education and Science is preparing a Law for Science, and no major science policy decisions are likely until that is approved. The draft now circulating suggests a new structure for the main research institutions which will include the replacement of CSIC by a new body called the Instituto Nacional de Investigaciones Científicas and the provision of more new posts. But even if the bill is eventually passed, its effects are unlikely to be felt for a year or two.

Pedro Puigdoménech

Soviet earthquake

Early warning system works

LAST week's earthquake in the Bukhara *oblast'* of Uzbekistan, which devastated the gas town of Gazli, involved no loss of life, although there were more than 100 injuries. But pumping operations at the main gas compressor, which feeds two trans-continental gas lines, were interrupted for a few hours.

The Uzbek authorities are justifiably proud of their rapid recovery from the disaster, and other seismic early warning system which enabled them not only to alert the public but to plan relief operations in advance. Within a few hours of the earthquake, tents, clothing and food had been rushed into the stricken area, and within 48 hours gas production from the Gazli field was back to normal.

The earthquake of unusual severity — barely a single building in Gazli was left intact even though, since the Tashkent earthquake of 1956, building regulations in Uzbekistan have required that all new buildings should be proof against shocks of 8–9 points on the Soviet 12-point scale.

During recent years several major research projects in the seismic areas of the Soviet Union have been aimed at methods

of forecasting earthquakes. These include the detailed mapping of known or suspected seismic areas, using palaeo-seismic evidence in the areas where no historical records are available. Networks of seismic forecasting stations have been set up in vulnerable areas, and major engineering works are carefully monitored for seismic consequences.

Uzbekistan's early warning system relies in part on historical records for the region. Typically, a major earthquake is preceded by a number of weak tremors. In January this year, frequent though weak tremors were recorded in the Fergana valley by a special expedition rushed into the area and a major earthquake occurred on 18 February. A 6-point tremor in western Turkmenia on 22 February was not considered to be related to the Uzbekistan shocks but, according to Dr Nikolai Sheabin, head of the Strong Earthquakes Laboratory of the Institute of Terrestrial Physics of the Soviet Academy of Sciences, was linked instead with the Kumdag earthquake of spring 1983 and even with the Krasnovodsk earthquake of 1895.

Vera Rich

Biological ethics

Nakasone takes the lead

Tokyo

THE first international conference on life sciences and mankind ended in Tokyo on 22 March with a set of conclusions to be presented to the heads of the Western industrial nations countries at the London summit in July.

The conference grew out of the proposal made by Japan's Prime Minister, Yasuhiro Nakasone, at last year's Williamsburg summit that problems presented by the rapid advance of the life sciences should be examined by an international committee. Nineteen scientists, theologians and philosophers — among them François Gros (Collège de France), Sydney Brenner (director of the Laboratory of Molecular Biology, Cambridge), Benno Hess (vice president, Max-Planck Society) and Sir Stuart Hampshire (Wadham College, Oxford) — were chosen to represent the seven summit countries and invited to visit Japan. In opening the conference, Mr Nakasone spoke of the growing public concern over *in vitro* fertilization, artificial determination of the sex of embryos, euthanasia, . . . and the treatment of those in a persistent vegetative state. The conference did not, however, succeed in solving the problem on the spot. Indeed, given that only a week earlier Pope John Paul II, speaking at a conference on Gregor Mendel at the Vatican, had warned genetic engineers against the "moral misuse" of their abilities and reinforced Roman Catholic opposition to techniques that allow fetal abnormalities to be detected, it would have been hard for universally acceptable ethical principles to be spelled out. All the conference could do was to acknowledge that the "more the life sciences are applied, the more they would bear on ethics, customs, politics and law" and that "critical problems might arise in the future" but "no agreement on specific (ethical) norms could be attempted at present". Emphasis was, however, given to the need for scientists to make their discoveries much more "understandable to the public" and to encourage discussion through education and the media.

Conference participants saw their main contribution in setting a precedent for a group of independent scholars, not representing their individual governments, to discuss major scientific issues and directly to advise heads of state. Deliberations are to continue next year, this time with the sponsorship of the French Government. The exact format has yet to be decided but it is hoped that attention will shift to specific problem areas and that representatives from outside the summit countries — particularly from the developing countries — will be permitted to attend.

Alun Anderson

Argentiniens regroup

SIR — With the coming of democracy in Argentina, Argentine research workers have organized their professional association with the aim of “building a science and technology engaged with the reality of the country and contributing to its effective independence and sovereignty”. In the past, a similar society functioned for researchers of the CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) but this was dissolved in 1974 and the members of the board of directors were prosecuted and dismissed from their jobs.

The new association, Asociación Argentina de Investigadores Científicos y Tecnológicos (AADICYT), is open to researchers from all disciplines belonging to universities, institutes, public or private enterprises, CONICET, and so on, and its membership covers all categories of investigators, including new fellows.

The president is Dr Enrique Segura, head of a neurobiology research laboratory of CONICET, and the two vice-presidents are Guillermo Dussel, a physicist at the National Commission of Atomic Energy, and Alberto Solari, professor of the Faculty of Medical Sciences. The secretary is Celina Lértora Mendoza, a philosopher, and the treasurer is Osvaldo Gosman, a mathematician and professor of the University of Belgrano.

The first objectives of the society are directed towards reinstating scientists and professional people who have been dismissed for political or discriminative reasons, whether still in this country or abroad. Also, to appeal to all Argentine investigators who are settled permanently in other countries to collaborate in this effort for the recovery and development of science and technology in Argentina. Various forms of collaboration are suggested: to accept fellows from Argentina in their institutions; to give courses and counselling in Argentina; to engage foreign institutes in cooperative programmes with similar institutions from Argentina. AADICYT is also asking that the authorities in science and technology accept delegates from our association in committees dealing with the institutional organization of science, with the investigation of irregularities, with mechanisms for the democratic participation of the researchers, and with a bylaw of rights and duties of research workers.

The address of AADICYT is given below. Our representative in Europe is Mariano Levin, Institut de Pathologie Moléculaire, 24 rue du Faubourg St. Jacques, Paris XIV, France. Collaborations and suggestions are welcome.

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India's home front

SIR — Self-congratulation is not the prerogative of artists. Scientists, especially Indian scientists, are adept at praising themselves, albeit subtly. In Dr Balasubramanian's letter (*Nature* 307, 312; 1984) the statement that there are “pockets of brilliance” in India, as in any other country, conveniently ignores the embarrassing questions about who is to decide who is brilliant and what constitutes brilliance and gives way to anthropocentricity. It is often forgotten that the greatest “pocket of brilliance” in recent times was not a research institute, but a Swiss patent office. Perhaps the greatest boon of this century is the fact that Albert Einstein was not born in India.

The recent spate of news concerning Indian science and scientists (*Nature* 304, 300; 1983: 4, 100, 307; 1984) tends to support Dr Malviya's contention (*Nature* 306, 10; 1983) rather than Dr Balasubramanian's perspective of science in India. On a recent visit to Indian universities and research institutes, I was appalled at the lopsided funding and facilities present there. Glamour in science rather than research relevant to India's needs guides the over-funded institutes. As a scientist Dr Balasubramanian should realize that comparisons of brilliance can be made only when all other variables are constant.

Dr Balasubramanian continues his self-congratulation in the form of lists (reiteration of what every Indian scientist has been echoing) of accomplishments of Indian scientists. I would not go to the extent of suggesting what some foreign based Indian scientists have been saying: these accomplishments have been made, not because of, but in spite of Indian scientists. Instead of listing the numerous accomplishments, Dr Balasubramanian should have asked “Is this all we have done in the nearly four decades of India's independence?”

I do not agree with everything that Dr Malviya says about Indian science and scientists. Leaving aside the competence of the people who control scientific circles in India, which Dr Malviya questions, I would like to point out that they, at least, have long enjoyed social position and freedom from criticism. Dr Balasubramanian's appeal to stop self-flagellation is nothing but a plea to maintain the *status quo* in Indian science. Science, of all human activities, requires criticism to make us aware of anthropocentric prejudice. I therefore applaud Dr Malviya's attempt at criticism of Indian science and would like to suggest that science governing bodies in India establish an institute and a journal devoted to constructive criticism, where not only scientists but also intelligent taxpayers can debate.

Incidentally, Dr Balasubramanian, pointing out that the issue that carries Dr Malviya's letter also carries an article by

Indian scientists working in India, neglects to mention that the authors of that paper thank another Indian abroad “for the many gifts that made this study possible”.

MANOJ MOJAMDAR

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SIR — The merit of India's science has been the subject of heated discussion in your correspondence columns. It is a healthy sign that such issues are raised now. A few years ago this would not have happened. Something good will eventually come of the polemics and accusations. The focus must, however, be on self-realization, an agonizing reappraisal at that and not indulgence in whipping by “prodigals” (Dr Malviya) or self-aggrandizement by the “natives” (Dr Balasubramanian): the present “natives” were once “prodigals” themselves.

As for the hostility of the natives towards the prodigals, I can confirm from my own experience. A few years ago, when I visited India for a family reunion, I offered to give a couple of seminars in two important institutions in Madras and Bangalore. The chairman of the chemistry department at one of them replied that in view of their hectic institutional activities, he was “unable” to “host” my seminar. He probably thought it was a favour to me and that I might ask for a job in his department! I subsequently gave a seminar in another department in the same institution without ever meeting the chemistry department chairman.

There is also general apathy among higher-ups towards their “subordinates”. There seem to be no peers among scientists in India — just power brokers and the rest. A few years ago, a prominent Indian expatriate visited India. One of the top science bureaucrats made arrangements to invite him to his office through a back door on the pretext that a lot of others for whom he had no time were waiting to see him at the front door. A lot has to change in the Indian science scene.

Nevertheless, Indian science has made significant progress in the past 35 years. I see good work coming out of the National Chemical Laboratory in Poona in the field of biotechnology, for instance. However, the achievement is minuscule compared with the potential. The myth of India being the third “superpower” in science when one considers the number of scientists and technicians has to be denied. As your correspondent, Vera Rich, observed in a recent report in *Nature*, the scientific force in India is not as awesome as it is made out to be when one considers the quality. But India has the potential. It has to be properly tapped.

Before the Indian government plunges into any bold venture, such as a “science

city", it would be well advised to assemble an international commission composed of 20 members to be drawn equally from the four following groups: (1) science leaders in India, (2) prominent Indian expatriate scientists in the United States, (3) leaders of scientific establishments in Europe, and (4) science educators and researchers in the United States. The task should be to make proposals which will improve science education in Indian universities, enhance the research environment in the universities and institutes, and develop support facilities such as manufacturing and servicing state-of-the-art instrumentation. I am sure that scientists from the United States and Europe would be glad to participate in a summer "sabbatical" of this type in the interest of world science.

S. SUBRAMANIAN

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SIR — Although we feel that there is nothing wrong in India's plans to build a technology city (*Nature* 305, 350; 1983) similar to the Tsukuba science city in Japan, we believe that this would not solve the main problem — India should stop deliberately encouraging its experts to leave the country. We agree with the suggestion (*Nature* 306, 310; 1983) that in order to encourage intellectuals to remain in India, money must be spent on improving the existing facilities for research. In addition, we suggest that India should make further provisions to send its young and talented scientific staff abroad for advanced training, and provide them with suitable facilities on their return. We understand that many scientists who go abroad are forced to resign their positions at home because of stringent governmental policies. When better conditions for research are available to the many intellectuals who remain in the country, India can stop recalling experts from abroad.

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Submarines risky?

SIR — The proposal to restrict nuclear arsenals to the seas where they can carry out their stated function of deterrence (*Nature* 23 February, p.680) has much to commend it, but certainly needs additions to cover antisubmarine warfare. Research in this field would have to be prohibited and those systems already established be dismantled, as they threaten to destroy the very invulnerability of submarines on which the whole proposal is based.

ROBERT WALL

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PWR in haste

SIR — In his article, "Next reactor still not decided" (*Nature* 26 January, p.307), your correspondent states that the Central Electricity Generating Board plans to build a pressurized water reactor (PWR) at Sizewell in "a record 19 months".

Would that such miracles could happen. Clearly his figure is a misquote for the 90 months from laying main foundations that CEGB is estimating for investment appraisal purposes for the construction of Sizewell B.

However, this still would not be a record. Our Magnox nuclear stations were in commercial operation in a shorter period, and some US PWRs and may French ones have been built in less than 90 months. Indeed, CEGB also has set itself a construction target of less than 90 months.

The article concludes with a misleadingly pessimistic picture of developments in the project. General inflation has, of course, resulted in costs rising in market price terms. But, so far, there has been no increase in our total estimate of the cost of Sizewell B in real terms (that is, at March 1982 price levels).

Although the length of the public inquiry means that we cannot place main foundations in line with the original timetable, this does not affect the 90-month construction period. It does, however, mean that the electricity consumer will have to wait longer for the economic benefits CEGB believes that Sizewell B, if approved, would bring.

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Not so simple

SIR — In your issue of 1 December (*Nature* 306, 418; 1983) you reproduced a year-by-year plot of Norwegian tobacco consumption for 1950–1980, presumably intended to support the argument that a ban on cigarette advertising can be effective in limiting the growth of consumption. The graph seems to be open to question on two grounds. One is that the rising trend changes to a decline before the year (1975) when the act banning cigarette advertising came into force. The other is the implication that the almost linear increase in consumption between 1950 and 1970 was caused by advertising. It would be prudent here to investigate the role of obvious economic variables such as prices and incomes. I have no data on tobacco prices in Norway, but a rough replotting of *per capita* tobacco consumption against *per capita* income at constant prices shows that the ratio between them had been declining since 1950, and against this background the further decline since 1970 looks much less striking. Such questions should be investi-

gated by standard econometric techniques before policy conclusions are drawn from *prima facie* correlations. The case for discouraging tobacco consumption on health grounds can only be weakened by opportunistic use of incomplete statistics.

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Paid referees

SIR — A recent letter (*Nature* 2 February, p.408) drew attention to page charges in journals and suggested a possible credit system for referees. However, according to an advertising circular recently received by me, at least one new journal (*Engineering Computations*) is adopting a "rigorous, remunerated reviewing policy". Apparently, this is in the hope that payment to referees will minimize the time taken to review the manuscript, thus ensuring rapid publication. I feel that this is a false premise on two counts. First, an examination of the received, revised and publication dates of many papers reveals that the delay between acceptance of a manuscript and publication is often longer than the time between submission and acceptance, which includes the time taken by the author to revise the manuscript. Second, journals, including *Nature*, achieve rapid publication without resort to payment of referees.

The adoption of remunerated reviewing policy by any journal can only be a retrograde step which may be interpreted by some people as further evidence of scientific integrity being sacrificed by financial gain. Also, it can only further increase the financial load imposed on individual scientists and libraries. Let us hope that the day never comes when a person can state his or her occupation as "professional journal referee"!

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Laughing matter

SIR — I am compiling an anthology, "Science with a Smile", and should welcome contributions of humour in the sciences: physics, chemistry, astronomy, mathematics, earth sciences, life sciences, and computer science — historic and contemporary. Appropriate would be anecdotes, biographical notes, cartoons, parodies, verse, examples of self-deception and hoaxes. I especially seek pieces which, while humorous, also have value in the history of science, providing insight into changing attitudes and personalities.

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The creationist challenge to science

Thomas H. Jukes*

The creationists want equal time for nonsense, and also want to eliminate current methods of teaching evolution and other sciences.

A CONTEST is in progress between creationists and supporters of science. It involves the teaching of biology, the funding of education, and the content of school science textbooks. It is not a polite discussion of differences in philosophy¹, and creationists are not merely desirous of "equal time" for their ideas in the classroom. They regard evolution as being inimical to their concepts of public education², and they perceive evolution as being diabolical³. Creationists seek debates to challenge evidence for evolution, but they refuse to discuss the religious nature of "scientific" creationism^{4,5}. Marsden¹ overlooks or omits the real nature of the creationist attack on science, and instead he has coined the term "anti-supernaturalistic mythology" to describe evolutionary science. He thus distorts the scientific principle, which is objectively searching for explanations of observations made in the laboratory or perceived in nature, without resorting to hypotheses based on magic or myth.

The Great Flood

The largest creationist organization in the United States, the Institute for Creation Research (ICR) in San Diego, is headed by a hydraulic engineer, Henry Morris, PhD, who, as befits his background, is fond of making up theories about the Great Flood. He is treated sympathetically by Marsden¹. However, Morris professes that the Great Flood "presents a new and powerful system for unifying and correlating scientific data bearing on the Earth's early history"⁶. He also suggests that fauna under Noah's care were kept in torpor by "divinely-ordered genetic mutations experienced by the individuals selected for preservation in the Ark, equipping them with the capacities for migration and hibernation"⁷. Inconsistently, elsewhere he says that all mutations are deleterious and therefore evolution cannot happen⁶. He also states that evolution is made impossible by the second law of thermodynamics, which will be repealed when the

time is ripe, following which the Earth's needs for water will be fully satisfied by the "pure river of water of life", another touch of hydraulic engineering. Morris denounces evolution intemperately: "The so-called geologic ages are essentially synonymous with the evolutionary theory of origins. The latter is the anti-God conspiracy of Satan himself"⁸. He invents theories of cosmology: one of them states that the stars may be involved in heavenly warfare, including particularly the planets and asteroids "in view of the heavy concentration of angels both good and bad around the planet Earth" and that these angels "can in many cases cause or prevent physical catastrophes on Earth and in the heavens"⁹.

The catalogue of ICR¹⁰ says that all genuine facts of science support the Bible, and that "scientific creationism should be taught along with the scientific aspects of evolutionism in tax-supported institutions" (p.12), that "each of the major kinds of plants and animals was created functionally complete from the beginning and did not evolve from some other kind of organism" (p.13), that "all things in the Universe were created and made by God in the six literal days of the creation week" (p.14), that all other men and women are descended from Adam and Eve, that unbelievers "must ultimately be consigned to the everlasting fire prepared for the devil and his angels".

Second in command of ICR is Duane Gish, PhD, University of California, Berkeley, 1952. His activities as Morris's protagonist have been likened to Thomas H. Huxley's role as "Darwin's bulldog". It is over-imaginative to compare Morris to Darwin, but, nevertheless, Gish peripatetic and tireless, glib and unabashed, has elevated himself into prominence by incessantly reciting a rehearsed attack on evolution. In so doing, he poses as an authority in several fields, but says¹¹ that "the Creator used processes that are not now operating anywhere in the natural Universe". When confronted with scientific objections to supernatural scenarios, Gish says that his religion is being attacked (see below). Gish asserts that "scientific crea-

tionism" is indeed science, but Judge Overton⁵ noted that Gish also said:

Creationists have repeatedly stated that neither creation nor evolution is a scientific theory (and each is equally religious).

Gish, a biochemist, sees a biochemical proof of creation in the defence mechanism of the bombardier beetle, *Brachinus* sp. He says¹² that the beetle "mixes up two kinds of chemicals, hydrogen peroxide and hydroquinone, . . . Now the marvellous thing about this — if you or I went to a chemistry laboratory and mixed up these two chemicals — BOOM! we would blow ourselves up." Gish then discourses on the beetle's need for an inhibitor and anti-inhibitor to control and time the explosion. These, says Gish, are components of a system that is too complex to have evolved, because the ingredients thereof would have destroyed the beetle before the complete apparatus had evolved. Emboldened by this mental victory, Gish invents 30-foot-long dinosaurs that had "a similar kind of chemical apparatus, or perhaps something even more fearsome" in the "strange, hollow, bony structures on the top of their heads, connected by tubes to their nostrils", as in the case of the *Parasaurolophus*. These, he says, were the fire-breathing leviathans described in Job 41:19-21.

Unfortunately, when hydrogen peroxide is poured on hydroquinone, the mixture slowly and quietly turns brown. Although he knows this¹³, Gish continues to recite before audiences the fable of the chemicals that go "BOOM!" Sale of his book¹² uncorrected, continues.

On 9 April, 1982, Gish spoke on "Creation versus Evolution" at the University of California, Berkeley before an overflow audience. His address consisted mainly of attacks on evolution. During the question period, Steve Obrebski, PhD, a marine biologist noted that Gish had neglected to mention that there are series of fossils that show linear transitions, and that Gish had not pointed out that the punctuation-equilibrium theorists have been criticizing neo-Darwinian theory rather than challenging evolution. Obrebski then said (my transcription from

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Gish's appearance at Berkeley:

I find it very disturbing that someone such as you would get up and make such statements in such a partial way and not really indicate to the audience that we are doing — those of us that are scientists or evolutionary biologists — precisely what science requires, and that is arguing contending hypotheses, presenting data and observations and analyses on contending hypotheses, and attempting to approximate the truth about laws of nature. Now . . . the director of your institute has claimed that there are concentrations of good and bad angels around the Earth, that UFOs are manifestations of the Devil or the dark forces, that our inanimate souls can travel faster than light, that although stars can't affect us, the evil spirits associated with them can, and that the scars on Mars and the Moon, the asteroids and the rings of Saturn and meteor showers are a reflection of some big turf battle between Satan and Michael and the angels. And you have said yourself that there was no problem of incest among the progeny of Adam and Eve because God made them genetically perfect. Now I ask you sir . . . what are the facts? How would you disprove them? Where do you get the material evidence for those facts, and how can you claim to be doing science when you make such wild and tentative assertions and then come in here and mix both sciences and mix up what the evidence is and claim that you are doing creationist science?

This was followed by prolonged applause in the form of a standing ovation.

Gish's response was, first, to make a diversion, alleging that someone else wrote the booklet, "Have You Been Brainwashed?" (Fig.1), published under his authorship¹⁴, and then to say:

I have not gone into this matter of religion. I have stuck strictly to science here tonight. It is always the evolutionists who want to bring in the matter of religion. This man has been ridiculing the Christian faith.

The connection between the Christian faith and the fantasies of Henry Morris is dubious, and, of course, it is the creationists who bring in the matter of religion: Gish in his booklet¹⁴, threatens his recalcitrant readers with terrible judgment and says, "I urge you to make your peace with God today".

Noah's Ark

Biology at ICR is headed by Professor Gary Parker, who has a doctorate in education from Ball State University. His specialties include the ecology of Noah's Ark, and the identification of fossil footprints. On the first topic, he has published *Dry Bones*¹⁵, described in ref.10 as having "strong evangelical emphasis, including the Fall, Curse, Flood, and promise of a new Earth.

The book describes the presence of a full complement of fauna, including dinosaurs, on Noah's Ark, which was "so big it could hold over 500 railroad boxcars full of animals with space left over". The precise number of boxcars is given at 522 by Whitcomb and Morris⁶. According to Professor K. Padian (personal communication), there were 300 genera of

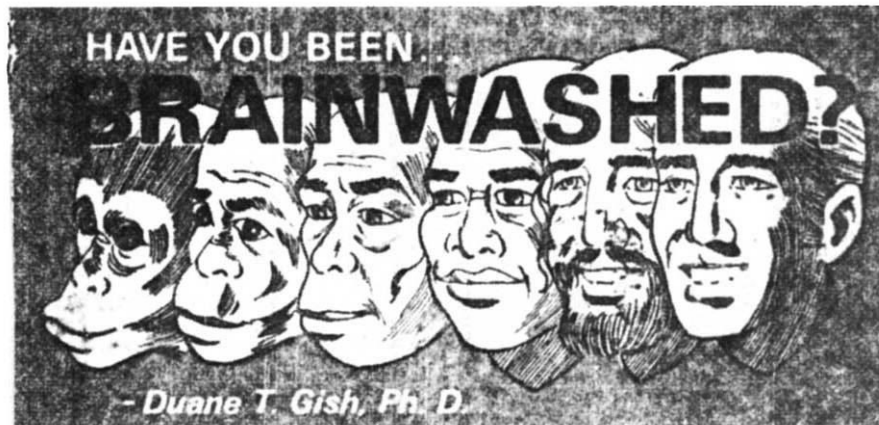


Fig.1 Cover of booklet by D. Gish¹⁴ showing disappearance of beard during human evolution.

dinosaurs, the species of most of which varied "from a small elephant to the weight of 30 or 40 elephants in size". Many were larger than boxcars. They ate a lot of food, which Noah must have had to provide.

Dry Bones aroused the ire of many parents when it and other creationist tracts were purchased with public funds and used in teaching science in a public school in Livermore, California. *Dry Bones* concludes with the following statement:

And we hope that you, too, will try to see God's world through God's eyes. The heavens declare his glory; the fossils show the power of His judgment.

Parker emphasizes simultaneous life of human beings and dinosaurs as evidenced by fossilized human footprints superimposed on dinosaur tracks. He has synthesized a fake exhibit to substantiate this falsehood (Fig.2), and he sends graduate students on "field research" for "measurements and analysis of human and dinosaur footprints formed contemporaneously"¹⁰.

Investigations by Professor Laurie Godfrey show clearly that these so-called human footprints are not authentic (a video cassette is available from L. Godfrey, Department of Anthropology, University of Massachusetts). Indeed, some of them are so oversized that the creationists have to fabricate mythical giants to fit them.

Harassment

A collegial relationship exists between ICR and the neighbouring enclave, the Creation-Science Research Center (CSRC). ICR is largely devoted to training future teachers who will deploy into schools in order to overthrow instruction in evolution. CSRC has a more urgent task, that of harassing the incumbents of the educational system. By this means, CSRC, in the words of its leader, Nell Segraves, hopes to acquire "50 per cent of the tax dollar used for education to our point of view"¹⁶.

Currently, CSRC is led by Mr Kelly Segraves, who wishes to ban a textbook, *Biology*, by Helena Curtis, from the public schools of San Diego. He requests the State of California to cut off "all state monies to the District of San Diego until this book is removed and the district once again

complies with the constitutional and legal restraints they have so flagrantly violated by the adoption, purchase and use of this book", which he designates as an offensive and an "illegal" book. The text¹⁸ was reviewed by 37 scientists and she had eight well-known scientific consultants. In the introduction, she stated, "I feel particularly privileged to be writing for young people. I like their curiosity, their energies, their imaginativeness and their dislike of the pompous and the pedantic". Not surprisingly, the book contains information on evolution. Segraves claims that this violates the decision of March 1981, in the trial in Sacramento¹⁶, in which Judge Perluss directed that the California State Board of Education re-circulate an earlier statement "that dogmatism be changed to conditional statements where speculation is offered for explanations for origins". But Judge Perluss also stated in his decision:

There is no contention here that evolution should not be taught in the public schools . . . that battle was fought and resolved by the Supreme Court of the United States.

Instead of confronting Segraves with this statement, the educational authorities in California have decorously fenced with him on questions of procedure, such as referring him back to the local school board. He has grown increasingly strident, and has threatened to file civil rights action for "withholding of all federal monies to the State of California" if this book is not "eliminated"¹⁷. Perhaps, however, his main interest is in selling his own books. One of these includes his own version of how the two kangaroos on the Ark found their way from Armenia across "the ruined Earth's surface" to Australia¹⁹. Such inventions would replace current information in school science classes if the Segraves family is successful.

Segraves, in November 1981, announced the programme of the Creation Creed Committee, organized by Reverend Robert Grant and himself. It announced that it would "review science textbooks and send hundreds of monitors into classrooms in many of California's 1,000 school districts to determine whether science teachers are violating the rights of Christian pupils in



Fig.2 Human being confronts dinosaur. Professor Gary Parker confronts camera. From ref.10.

public schools". The committee said it would run advertisements over Christian television stations and many radio stations throughout California, inviting concerned parents and others to alert the group to teachers and books they suspect may be violating the state policy. The committee advises supporters to remind school boards that failure to comply with their demands would lead to pressure for the withholding of county and state funds, and "the loss of teaching credentials for individual offenders". The committee is preparing education kits and a film for those who will be monitoring schools "so that parents of children can more intelligently evaluate what children are being taught". Segraves said that the committee will utilize resources of the 40,000 ministers and the 328,000 members of *Christian Voice* to conduct a nationwide campaign against exclusion of creationism from the classroom²⁰.

Censorship

Censorship of school science textbooks has been successfully practiced by creationists

for more than 50 years²¹. Currently²¹ many publishers are collaborating with alacrity and much of the censorship is practiced in Texas, which is the second largest textbook purchaser in the nation. The effect is nationwide, because publishers cannot afford to maintain two separate editions. A publishing executive is quoted as saying²² "You're not going to find the word 'evolution' in the new textbook *Experiences in Biology*. The reason for self-censorship is to avoid . . . controversy. We'd like to sell thousands of copies".

In Texas, until 1983, only those who objected to books could file complaints, and testify before the textbook committee. Those who wanted to defend a book or its ideas were excluded from the process. Censorship has become "big business", led by the Gablers of Longview, Texas, with a full-time staff of Educational Research Analysts^{23,24}. Recently, 28 of 49 textbooks attacked by the Gablers were rejected by Texas. They commented²⁵ "While the word 'evolution' was removed, the content is as evolutionarily dogmatic as ever and will serve to indoctrinate students

away from true scientific facts".

The Texas textbook proclamation, published by the Texas State Board of Education, contains the following paragraphs adopted May 1983.

Textbooks that treat the theory of evolution shall identify it as only one of several explanations of the origins of humankind and avoid limiting young people in their search for meanings of their human existence.

Each textbook must carry a statement on an introductory page that any material on evolution included in the book is clearly presented as theory rather than fact.

The presentation of the theory of evolution shall be done in a manner which is not detrimental to other theories of origin.

Surfeited with money from oil, the University of Texas is now proclaiming a laudable intention to expand greatly its commitment to science by erecting huge new buildings and luring famous professors to Austin²⁶. Of greater value would be a successful effort to combat the censorship of science textbooks, in which Texas leads and dominates the United States. Can the Longhorns meet this challenge? □

Correction

In my commentary "Just how objective is science?" (*Nature* 306, 727-730; 1983) I included Donald Goldsmith among astronomers who attempted to explain away Adriaan van Maanen's reported measurement of internal motions in spiral nebulae as an instrumental error. Dr Goldsmith informs me that my slipshod reading of his article turned his statement about a systematic error into my misinterpretation and subsequent gratuitous criticism of his article. I apologize to Dr Goldsmith and to readers of *Nature* for any error I committed in reading and reporting upon his article; I am happy to report that Dr Goldsmith believes that by far the most likely explanation of van Maanen's results is that he let his preconceptions affect his analysis.

Another reader has questioned why my article contained no citations of van Maanen's work. To save space, in the instances of all three twentieth century cases (rotation of spiral nebulae, general solar magnetic field and gravitational redshift) and, indeed, in the earlier cases as well, I cited only the historical studies and not the scientists' work. I regret any inconvenience this may cause readers, who consequently must first look up the historical paper cited to find the citations to the original scientific papers.

The reference given for Hale's graph of a solar magnetic field is actually that for van Maanen's paper on the rotation of M101; the correct reference for the Hale paper is *Astrophys. J.* 38, 27-98 (1913). The idiosyncratic features of Harriot's drawing of the Moon are better displayed in the article in *J. Hist. Astr.* 9, 117-122 (1978).

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Three dimensions for two

Cellular automata make it possible to produce some special solutions of the Ising-lattice problem in three dimensions. Will the hunt for general solutions now begin again?

THE title "Exact results for two and three-dimensional Ising and Potts models" will excite only a small community of physicists, but for them it will be eye-catching in the highest degree. For is this not the solution to a problem on which a host of people have broken their heads in the past forty years? That, certainly, is how it seems — but the title is a little misleading, almost like the headlines with which popular newspapers try to capture readers when the flow of news is slack. And if the title, that of a paper by Eytan Domany from the Weizmann Institute in Rehovot, Israel, but writing from Stanford University (*Phys. Rev. Lett.* **52**, 871; 12 March 1984), had more properly read "Some exact results. . .", whoever would have been sitting on the edge of his chair?

But even if the text of Domany's paper is something of a letdown, it is an interesting and potentially important development in the solution of what is probably best known as the Onsager problem. The problem was first made interesting (in the sense of being made tractable) by Lars Onsager in the 1940s. The Ising lattice, so called since the 1920s, is the simplest model there could be of a ferromagnetic material as well as of many other kinds of systems capable of order-disorder transitions.

The problem is to calculate the thermodynamic properties of a lattice in which each vertex is occupied by a physical variable which can have one of only two values, say -1 and $+1$, and suppose that only interactions between nearest neighbours in the lattice are significant. The energy of interaction between two neighbouring sites will depend only on the values assumed by the two sites. If the variables represent electron spins, the model may describe a ferromagnetic material. If they represent electric dipoles, the lattice will stand for a ferroelectric material (which is, of course, an ordinary dielectric above some critical temperature). If the physical variable represents one or other of two different kinds of atoms, the system likens that of binary alloys such as β -brass whose mechanical properties depend critically on whether there is or is not long-range order in the system.

Onsager's achievement was to demonstrate that it is possible to obtain exact solutions for the two-dimensional Ising lattice, at least with the help of seemingly undemanding assumptions such as that the properties of a finite piece of lattice are those of an infinite lattice in which the

finite piece repeats itself periodically. The importance of Onsager's innovation, essentially a neat piece of algebra, was that it was the first exact calculation of the thermodynamic properties of a system in which phase transitions might be expected. Earlier treatments of the thermodynamics of phase transitions, that of Landau for example, entailed expansions of the free energy of each of two phases in terms of some small quantity (say the difference between temperature and transition temperature) and were thus unable to describe the behaviour of thermodynamic quantities arbitrarily close to the transition.

So, forty years ago, it seemed natural to suppose that the problem of phase transitions had been solved once and for all. Onsager's original solution was for a two-dimensional square lattice. He and G. H. Wannier soon afterwards provided a solution for a triangular lattice and, interestingly, showed that that was the equivalent to the solution for a hexagonal lattice obtained by drawing a line perpendicular to each triangular side through its mid-point. (The thermodynamic properties of the "inverse" lattice are those of the original provided that the temperature is inverted by the critical temperature.)

In reality, experience has been frustrating. A variety of two-dimensional lattices have been solved by variants of Onsager's technique or by other means. The model has been generalized in a variety of ways — the Potts model is, for example, the generalization in which each vertex of a lattice is occupied by some analogue of spin that can assume more than two values. The Ising lattice has also, quite unexpectedly, been the starting point for A. H. Wilson's demonstration that the renormalization of a gauge field theory can be represented by the calculations of the properties of a suitably chosen lattice. But the three-dimensional Ising lattice has never been exactly solved by Onsager's method or any other. People have had to be satisfied with numerical calculations (which nevertheless show that, for example, phase transitions on a three-dimensional lattice do involve a finite change of heat content, as observed when cooling iron through its Curie temperature). It has even been suggested that the three-dimensional problem is inherently insoluble.

That it should be difficult is easily appreciated. To calculate the thermodynamic properties of even a model system, it is necessary to enumerate all

possible particular values of the energy. The next step is to construct the partition functions for the system, the sum over all possible energy states E_j of the quantity $N_j \exp(E_j/kT)$ where k is Boltzmann's constant, T the temperature and N_j is the number of configurations with energy E_j . The thermodynamic quantities are then simply derived — the entropy, for example, is simply proportional to the logarithm of the partition function. The snag is that evaluating the quantities N_j is an awkward combinatorial calculation. Onsager's two-dimensional trick was to make the partition function factorize.

So how can Domany have made progress even with some particular examples of a three-dimensional lattice? His trick is to combine the known solution of a two-dimensional hexagonal lattice with another combinatorial kind of calculation, the technique of cellular automata popularized (after J. von Neumann) by Stephen Wolfram (see *Nature* **305**, 469; 1983). That is a means by which the evolution in "time" of an array of objects may be simulated by machine once rules have been specified for the dependence of the members of one "generation" on those of the predecessor generation.

The advantage of starting with a hexagonal Ising lattice is that when the interactions between neighbours are such that the whole system is ferromagnetic, the lattice can be considered as if it were two interpenetrating triangular lattices inverted with respect to each other. The rules for the time evolution of such a system considered as a cellular automaton make it possible to regard each successive "generation" in the calculation as another layer of a three-dimensional lattice. But the outcome is, as followers of Wolfram might expect, not a three-dimensional lattice of identical planes but one in which hexagonal and triangular arrays alternate as the structure known as hexagonal close-packed.

The same trick works with the Potts model — starting with a hexagonal lattice in two dimensions, Domany is able to generate a solution for a hexagonal close-packed lattice in three dimensions. For the time being, the interest of much of this argument is that it should be possible to ground at least some of the conjectures based on numerical calculations in analytical results. But nobody should be surprised if the old Adam — the hunt for a general three-dimensional solution — does not begin all over again. **John Maddox**

Developmental genetics

A common segment in genes for segments of *Drosophila*

from Michael Akam

THE beginnings of the molecular analysis of homoeotic genes, which seem to control segment determination in the fruit fly *Drosophila melanogaster*, have raised hopes that at long last an understanding of the relationship between the genetic information an individual inherits and its three-dimensional form may be in sight (see ref. 1 for a brief review). Adding to those hopes, Walter Gehring's group report in this issue of *Nature* (see p. 428) that each of the two distinct homoeotic gene clusters contain several copies of the same short sequence of DNA, dubbed 'H', of which there are only about eight copies in the whole *Drosophila* genome². The implication is that this sequence must have some crucial role in the actual process of segment determination.

There are two clusters of homoeotic genes in *Drosophila* genome — the bithorax (BX-C) and Antennapedia (ANT-C) gene complexes, the former being more concerned with the determination of thoracic and abdominal segments and the latter with thoracic and head segments³⁻⁷. How many genes there are within each complex is not yet clear. Lewis suggested that BX-C contains nine genes, one regulating the development of the third thoracic and each abdominal segment². But it now seems likely that both the functional organization of genes within the complex and the relationship of genes to segments are more complex than this. For example, also in this issue (see p. 454), Struhl shows that BX-C can be split by chromosomal rearrangements into two widely separated

but fully functional domains⁸. One of these, the *Ultrabithorax* (*Ubx*) domain, is required principally in the thorax; the other in the abdomen. The realms of action of these two domains do not, however, meet at a segment boundary, but within the first abdominal segment, probably at the lineage boundary defining anterior and posterior compartments.

In ANT-C, two genes, *Antennapedia* (*Antp*) and *Sex combs reduced* (*Scr*), are clearly involved in the regulation of segment identity^{6,7}. *Antp* products are required in thoracic segments to repress the development of head structures; when they are inappropriately expressed in the head, antennae are converted into leg structures (see the figure). *Scr* products are required specifically in the labial and prothoracic segments. A third homoeotic gene, *proboscipedia* (*pb*), is separated from these two by at least three other genes, mutations in which give rise to phenotypes which are not obviously homoeotic^{5,7,9}.

Chromosomal sequences from both BX-C and ANT-C have been cloned¹⁰⁻¹², but only the *Antp* domain of ANT-C and the *Ubx* domain of BX-C have been characterized in any detail. Whereas most *Drosophila* genes are compact, with short introns, these two genetic units are exceptional, but similar, in containing very long transcription units (*Ubx* is about 75 kilobases (kb), *Antp* more than 100 kb) which give rise to several different species of processed RNAs by the removal of large introns.

Despite the functional analogy and

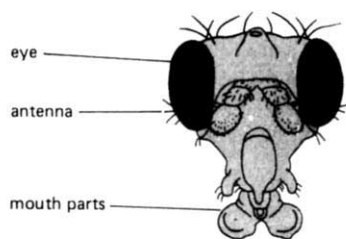
structural similarity between these two units, and despite claims made with the benefit of hindsight, few if any of those working with *Drosophila* homoeotic genes predicted what McGinnis *et al.* have found — that the *Antp* and *Ubx* genes contain a common, short sequence. As it happens, the first repeat of the H sequence to be identified did not immediately suggest any functional correlation between the H repeat and homoeotic genes. Both research groups cloning the *Antp* region noticed weak homology between what is now recognized as an H sequence and a cloned fragment located 40 kb away on the chromosome and within a short transcription unit which coincides with a cluster of *fushi-tarazu* (*ftz*) mutations^{11,12}. The phenotype of *ftz* mutations, though striking — embryos homozygous for *ftz* mutations develop only half the normal number of segments but of double the normal size, is not homoeotic in any accepted sense.

But the H repeat also hybridizes to a small number of other fragments within the *Drosophila* genome — a total of eight clearly resolved *Eco*RI restriction fragments. By good luck, or insight, McGinnis and his colleagues in the Gehring laboratory in Basel chose to screen the available clones from BX-C for the presence of one of these fragments, and found one located in the 3' exon of the *Ubx* transcription unit, a position analogous to that of the H repeat in the *Antp* gene².

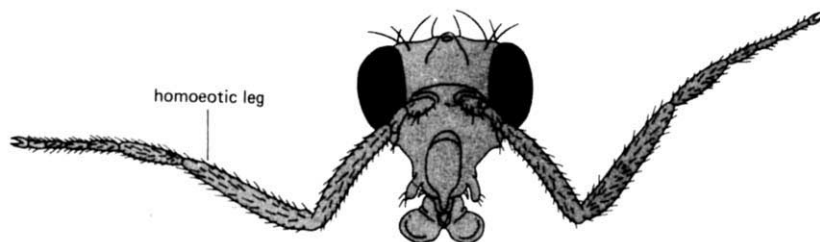
Where, then, are the other five or more copies of the H repeat; are they also within homoeotic genes? Using clones selected from a genomic library by their homology to both *Antp* and *ftz* (and thus to the H repeat), the Basel group identified two chromosomal sequences, in addition to *Ubx*, which contain the H repeat. One of these, represented by clone p93, hybridizes to chromosomal bands at 89E, the site of BX-C; the other, clone p99, to a site within or very close to ANT-C.

Clone p93 hybridizes to regions of BX-C beyond the *Ubx* domain, which have been cloned, though not yet fully characterized, by Bender and his colleagues at Harvard University. That places the H repeat of p93 approximately 130 kb from the copy in *Ubx*, in a region in which mutations have no effect on thoracic segments, but transform abdominal segments^{3,4}. By hybridizing p93 to size-fractionated RNA and to RNA in sections of embryos, the Basel group shows that the p93 clone is homologous to a complex set of transcripts which are located in the posterior abdominal segments of the embryo. There is little doubt therefore that the p93 clone is derived from a gene in the abdominal domain of BX-C.

The identity of the gene carried by p99 is less clear. The chromosomal region to which it hybridizes lies beyond the end of current chromosomal walks in ANT-C, in a region where only one of the three identified genes is clearly associated with hom-



The antennae on the head of *Drosophila* (left) are transformed into legs by an extreme example of a homoeotic mutation within the Antennapedia gene complex. (Modified from B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts and J. D. Watson *The Molecular Biology of the Cell*, Garland Publishing, 1983.)



oeotic mutations. However, here again the H repeat is present within a transcribed sequence. In the blastoderm, the transcript accumulates specifically in the cells of a region corresponding to the primordia for segments of the head and mouthparts. While not conclusive, this strongly suggests that the p99 clone is identifying a homoeotic gene within ANT-C.

In summary, all five identified H repeats lie within transcripts which show strikingly localized patterns of expression. Three at least are within homoeotic genes, two of these within units of BX-C and one at ANT-C. The repeat at *ftz* appears not to lie in a conventional homoeotic gene, but the *ftz* gene itself may actually be embedded within the *Scr* locus, an overly homoeotic gene of ANT-C (refs 11, 12).

What is the function of the H repeat? McGinnis *et al.*² cite unpublished sequence data to argue that it encodes a conserved protein sequence, present in the same orientation relative to transcription in at least the *Ubx*, *Antp* and *ftz* genes. This is the first real evidence for the appealing, and simplifying, suggestion that the genes which specify segment identity in the different regions of the embryo are performing related biochemical functions.

Lewis suggested that the genes of BX-C evolved in parallel with the diversification of the thoracic and abdominal segments of insects³. By analogy, the *Antennapedia* function would date from the origin of distinct head and thoracic structures — a diversification at least as old as the arthropods. For the H sequence to have been conserved since then, stringent constraints must have been imposed by the need to retain the function, and hence the structure, of the protein encoded by it.

Perhaps this function evolved specifically to mediate some aspect of segment determination. If so, the H repeat should prove to be a characteristic marker of segment-determining genes. More probably, the function will prove to be an ancient one. Although characteristic of one family of homoeotic genes, the same protein may, in different gene families or in other organisms, be the means to very different biological ends. There will be many who seek to find out what its function is. □

Radiocarbon dating

Accelerating carbon dating

from R.E.M. Hedges and J.A.J. Gowlett

THE dates reported in this issue of *Nature* (pages 443 and 446) by groups from the University of Rochester¹ and the University of Arizona at Tucson² are among the first fruits of the method of radiocarbon measurement by accelerator mass spectrometry. The method, which uses the large tandem Van der Graaff accelerators found in nuclear physics laboratories, was first demonstrated as practicable in 1977 but has been a long time maturing. Now, several groups, notably from Chalk River³, Zurich⁴ and our's from Oxford⁵ in addition to those from Rochester and Tucson, have accumulated a sufficient number of radiocarbon dates, at a useful level of accuracy, for the archaeological impact of the new method to be felt.

The advantages of measuring carbon-14 levels in archaeological and environmental samples by mass spectrometry have been clear from the start. Since carbon negative ions can be produced with an efficiency of a few per cent (in terms of sample consumption) and subsequently transported, separated from neighbouring masses and detected with an efficiency of 25–30 per cent, the overall efficiency corresponds to that obtained by counting all the β -decay events from the sample for about 80 years. Accordingly, accelerator mass spectrometry should make it possible to use samples at least a thousand times smaller than in conventional radiocarbon measurement by β counting. With typical beam currents, a counting time of 30 minutes is sufficient for most archaeological samples. A further advantage is that there is no cosmic-ray background, and misidentification of the detected particles can be negligible.

The development of the method has been interesting. The obvious problem, that of detecting a ^{14}C nucleus in the presence of a 10^{12} -fold excess of ^{12}C , almost as much as ^{13}C and isobars such as ^{14}N and numerous molecular combinations, has turned out to be relatively straightforward, even at accelerator energies somewhat lower than originally intended. Nevertheless, ions are accelerated to an energy at which the principal charge state produced by a stripping collision is C^{3+} ; confusion by molecules is eliminated by analysing a high-charge state, while isobars can be distinguished by measurement of their energy-loss rates in the detector.

One major development has been the construction of purpose-built systems based on 'small' accelerators. The Oxford and Tucson groups work at a terminal voltage of 1.8 MV, achieved with a commercially packaged system in Tucson. The point of constructing a purpose-built system is to achieve an accuracy sufficient

to contribute usefully to the 50,000 or so dates, with an accuracy averaging about 1 per cent, already amassed by conventional ^{14}C dating. This accuracy seems unusually demanding to an accelerator physicist. Accordingly, most laboratories with existing large accelerators have turned their attention to other trace nuclides such as ^{10}Be , where the database is much more primitive.

The greatest hindrance to high accuracy comes in the comparison of the analysed beam of the unknown sample with that of a standard. The fine details of the distribution of the ion beam are sensitive to small changes in the conditions of the ion source, especially in a source of high extraction efficiency working with a solid target. Much work has gone into suitable target preparation methods; by now there are more preferred methods than groups. Methods vary in the type of sample chemistry needed, not least to contain background contamination by modern carbon, and in the ion source performance. Accurate dating beyond the range of conventional ^{14}C dating (35,000 years) needs reasonably high beam currents and contamination of the target by modern carbon at well below the microgramme level.

The oldest dates so far obtained are in the 40,000-year range. That contamination can be a problem, is illustrated by the apparent dating of the ideal target-source for carbon ions, supposedly ^{14}C -free geological graphite, to only 60,000 years.

Because of difficulties encountered in the development of accelerator dating, most of the applications so far published deal with issues of verification, which can usually be tackled even without high precision. Verification is often crucial to archaeological interpretation, which relies on assessing whether examples of domesticated plants or animals, or even of artefacts, are as old as they appear to be. The choice of samples for dating is heavily biased by the climate of expectation. Thus if grain is found in a Neolithic grain pit, nobody is likely to wonder whether it could be later Roman grain brought down by burrowing of rodents. But if, for example, domesticated grains are found on early sites where one would expect the economy to be hunting and gathering, as on some sites over 10,000 years old in the Middle East, very many questions will be asked. Dating may not be the only way to answer them, but it has the great advantage of settling matters beyond reasonable doubt.

The dating of the early human colonization of the Americas is beset by contextual problems of this kind. Although already suspect on archaeological grounds, the case for the Yuha burial being evidence

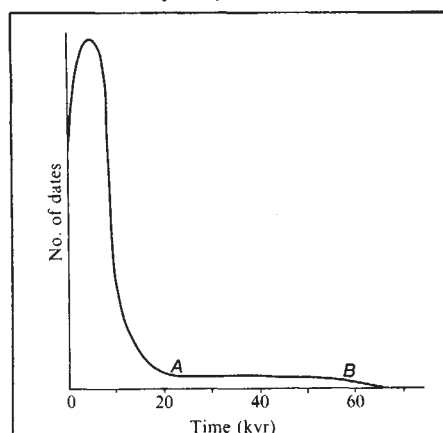
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of early colonization is effectively dismissed by the date of $3,850 \pm 250$ yr BP produced by Stafford *et al.*². (Evidence of early colonization still exists from much better documented sites, in South America and the eastern United States.) Conard *et al.* illustrate the value of the accelerator dating in studies of early domestication¹. They show that domestication of some crops started considerably earlier than generally expected in the north-central United States, although maize now appears to have been an unexpectedly late arrival.

It would be unfair to see all questions of verification as failures of ordinary archaeological methods. In many cases no amount of skill can determine whether small excavated objects have moved through sediments. Now that some groups can produce accelerator dates accurate to within 1–2 per cent, the technique can be extended to problems that are 'tight' in time range (for example, domestic cows 1,000 years 'too early', camels 1,500 years 'older' than yet accepted). Even now such applications are a minority of those competing for accelerator time.

Within five years, when world accel-



The approximate distribution of conventionally obtained radiocarbon dates, based on the output of some British laboratories over a period of several years, highlighting the scope for accelerator dating, for example at point A, where conventional dates tail off rapidly, as well as at B, where contamination poses serious difficulties for all radiocarbon dating.

ator capacity will exceed 1,000 dates per year, the main emphasis will be on straightforward dating of material which could not previously be dated, either because it consisted of very small objects, such as artefacts from the past 10,000 years or single bone or charcoal samples in the 10–50,000 years range, or because it was more than 50,000 years old. If the distribution of accelerator dating was to follow that established by conventional radiocarbon dating, the total impact in a five-year

period would be relatively slight (see diagram). It would be more desirable to lay equal emphasis on the periods before and after 10,000 BP. This strategy would almost double the number of dates for the period of 10,000–50,000 years ago, the critical period leading up to the appearance of modern man and then to the domestication of plants and animals. The question, then,

is not so much that of how accelerator dating can most successfully be applied as that of how it can best be rationed out to make the most useful contributions in a number of fields. □

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Huntington's disease

Charting the path to the gene

from Charles R. Cantor

FLUSHED with the success of the discovery by James Gusella and his colleagues of a marker on human chromosome 4 linked to the gene for Huntington's disease (*Nature* 306, 234; 1983), the participants at a recent workshop* in Los Angeles of the Hereditary Disease Foundation focused their attention on how to accelerate the search for the Huntington's gene itself and the progressive impact of the search on pre-symptomatic diagnosis of the disease.

Gusella *et al.*'s approach started from the fact that there is approximately a 1 per cent chance that any base in a chromosome of an individual will differ from that of another individual or will differ between an individual's two homologous chromosomes. Such differences, or polymorphisms, are inherited and will sometimes lead to differences in the pattern of cleavage of DNA by restriction enzymes. A polymorphism close to a deleterious gene will tend to be inherited along with that gene. Thus, tracing the inheritance of numerous polymorphisms, by the use of randomly selected cloned DNA fragments as probes, will eventually reveal a polymorphism close to any gene of interest. It was anticipated that an examination of a few hundred cloned fragments would be necessary to find one close enough to the Huntington's disease gene to be useful but Gusella *et al.* were remarkably lucky and found their polymorphism in the first 12 cloned sequences examined.

From the pattern of inheritance of Gusella *et al.*'s polymorphism, a fragment of DNA ignominiously called G8, one can calculate that it is 3 to 5 million base pairs from the gene. This is the length of all the DNA in a bacterium. It should not be too hard to narrow down the location of the Huntington's gene but since it probably represents less than 0.1 per cent of the chromosome fragment identified by G8 and since there is no hint of what the gene does or what it looks like, finding it by existing techniques will be an enormous task.

The next step, outlined by Gusella, will be to bracket the Huntington's gene with a

second marker, on the opposite side from G8. John Wasmuth (University of California, Irvine) described hybrids of human and rodent cells containing chromosome 4 as the only human chromosome which have allowed the construction of a library of cloned fragments from chromosome 4. This narrows the search for new markers but the inheritance of each one must still be traced through many related individuals.

To reveal which chromosome has been passed along to each child, it is necessary to have polymorphisms that are different on the two homologous chromosomes of an individual. The more polymorphisms one has, the more accurate the resulting picture of inheritance. Hence the value of electrophoresis in denaturation gradients, described by Lenard Lerman (SUNY-Albany), as a means to find all polymorphisms on a DNA fragment, not just those that lead to differences in restriction enzyme cleavage. Electrophoretic separations of DNA molecules as large as 2 million base pairs have been developed by David Schwartz and Charles Cantor (Columbia University). They will allow the G8 locus to be isolated as a very large DNA fragment and then subcloned. Hans Lehrach (EMBL, Heidelberg) described methods for scratching out a single piece of a mammalian chromosome and microcloning DNA from it. Once the approximate chromosomal location of G8 is known, this will provide other clones of near-by DNA regions.

Another approach is to construct stable rodent cell lines containing fragments of human chromosome 4 which include G8. One method would be first to use Richard Mulligan's (MIT) retroviral vectors to insert a selectable marker into human chromosome 4; then to produce a cell line containing the marker integrated near G8; and finally to transfect rodent cells with chromosomal fragments from that cell line using microcell methods described by Tracy Lugo (University of Southern California) or other methods described by David Housman (MIT). The selectable marker will enable a rodent cell line carrying the G8-containing fragment to be identified and maintained. Repeated sequences unique to humans will allow the

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*Gene mapping: clinical and scientific impact on neurogenetic diseases, Los Angeles, 7–8 January 1984. A full report will be published by the Hereditary Disease Foundation.

human DNA to be separated from rodent DNA for subsequent cloning.

Until Gusella's finding it would have been hard to motivate a coordinated effort to mesh these new techniques towards an objective as elusive as the Huntington's gene. However it is clear that the methods will complement and assist each other in providing DNA fragments closer to the gene. Eventually the techniques will provide a physical map of a few million base pairs surrounding the Huntington's gene and a complete set of clones representing all of the DNA in this region. Analysis of DNA polymorphisms will progressively identify those clones whose inheritance more and more closely matches Huntington's. Along with the physical DNA map this information will narrow the location of the Huntington's gene to less than a million base pairs.

Fragments nearer the gene may be identified through individuals who display evidence of recombination between a mapped marker and the Huntington's gene. This random exchange of DNA between two homologous chromosomes occurs roughly 1 per cent per chromosome per generation. As the search narrows, the number of individuals who must be examined to find an interchange between the closest marker and the gene multiplies rapidly.

At this stage, the extensive polymorphism of human DNA that revealed the G8 marker in the first place turns from an asset to a severe liability. For even if the search has been narrowed to 500,000 base pairs, polymorphism will insure that there are a few thousand bases that differ between any two individuals and only one of these differences is likely to represent the origin of Huntington's disease. Without knowledge of the function of the Huntington's gene or the nature of its product it will be impossible to tell which of the polymorphisms is the mutation. Perhaps luck will be on the side of the scientists again; without it, the rate of progress might slow considerably.

Since Huntington's disease is dominant, it is conceivable that expression of the mutant gene will produce neurological symptoms in other species. An animal model of Huntington's might be created by moving the human gene into a mouse. One approach would be to transfect a teratocarcinoma cell line with DNA containing the Huntington's gene and then to mix these cells with mouse embryos and derive chimaeric mice. Although this approach has many pitfalls, it is worth a gamble in view of the remarkable conservatism of brain proteins, as illustrated by Carol Miller's (University of Southern California) and Seymour Benzer's (Caltech) demonstration that more than half of a panel of monoclonal antibodies raised against *Drosophila* brain cross-reacted with human brain tissue.

If no animal model for Huntington's appears one is only left with brute-force

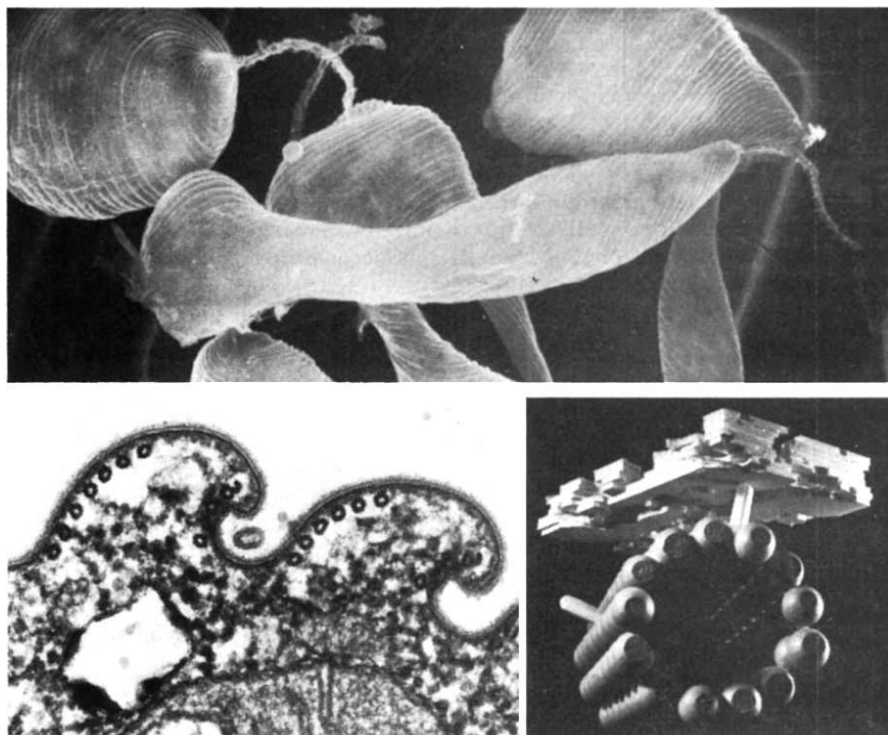
approaches. If Huntington's arose from a single mutational event which became distributed through different human populations, the DNA region immediately adjacent to the gene should be relatively devoid of polymorphisms and so, perhaps, detectable. Other strategies will require some consideration of biological function. A million-base-pair stretch of human DNA containing the Huntington's locus should code for about 25 proteins and Kelly Mayo (Salk Institute) described new ('open reading frame') vectors that should allow their coding regions to be identified and sequenced. Comparison of the sequences with those of genes for proteins of known function might help towards identifying which of the 25 proteins is the Huntington's protein. If this approach fails, one will be reduced to searching for abnormal expression of the genes in the neural tissues of Huntington's patients. There is no guarantee that anything would be revealed by such a search.

As the location of the gene becomes more certain, the accuracy and generality of presymptomatic and prenatal tests for Huntington's disease will increase markedly. The G8 polymorphism is only useful in some members of certain Huntington's families. Tests based on G8

are subject to some statistical uncertainty. Closer markers will eventually permit diagnosis of virtually all individuals with very little uncertainty.

The moral and ethical issues raised by this prospect are substantial. They involve potential conflict between parent and child if one person wishes presymptomatic diagnosis and the other does not. They involve potential conflicts between individuals and society given that every child of an affected individual has a 50 per cent chance of developing the disease. The fact that these issues are being seriously discussed underscores the current optimism about the progress that can be expected. It is, anyway, prudent to air the issues now, because Huntington's could serve as a model for many other genetic diseases. It is remarkable how many biological skills must be coordinated to optimize the approaches to such diseases. The degree of planning and organization is familiar to high-energy physicists but not to most biologists. It should turn out to be a very satisfying one. □

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LONGITUDINAL ridges trace a helical path along the length of the cell surface of the unicellular flagellated alga *Distigma proteus* (top). Sections through the ridges reveal microtubules underlying each ridge (below left) and probably involved in the euglenoid movement of the cells. Between four and nine microtubules are grouped under the smoothly curved border of each ridge, two more are at the point where the membrane folds inwards and makes a sharp bend and one or two are situated beneath the deepest part of the surface groove. Overlying the microtubules is a crystalline membrane lattice, three-dimensional image reconstruction of which shows a featureless inner surface and an outer surface of double rows of roughly pentagonal particles with a central depression. A model (below right) of the membrane lattice and a microtubule composed of thirteen protofilaments, includes microtubule-associated protein molecules (cylindrical rods) presumed to link the microtubules to the lattice. From J.M. Murray *J. Cell Biol.* 98 283 (1984).

Cell biology

Architecture of interphase nuclei

from Virginia A. Zakian

THE orderly appearance and behaviour of chromosomes during mitotic and meiotic cell division must reflect precise and regular three-dimensional associations in the dividing eukaryotic cell. Between divisions (interphase), chromosomes unfold and usually disappear from view, raising the question of whether interphase nuclei possess a specific and predetermined spatial organization. If they do, that organization might influence or even determine the patterns of RNA transcription and DNA replication during interphase. The second stage of an ambitious attempt by John Sedat and co-workers to probe the structure of the interphase nucleus is reported on page 414 of this issue.

Specifically, Sedat's group is investigating the arrangement of polytene chromosomes in interphase cells of the salivary glands of *Drosophila melanogaster* larva. These chromosomes are ideally suited for such studies: successive rounds of endo-replication produce huge chromosomes in which over 1,000 chromatids are exactly aligned and characteristic bands can be correlated with specific genetic loci. Moreover, transcriptional activation occurs at individual loci in a characteristic temporal sequence and can be visualized by the appearance of puffs at specific sites.

The studies of Sedat's group are carried out on intact unfixed cells, using procedures designed to minimize disruption of intracellular associations. Chromosomes are stained with DNA-specific fluorescent dyes and examined by fluorescent light microscopy using sophisticated computer-assisted methods. Twenty-four optical 'slices' are taken through each nucleus. An image-processing program handles focus control and the averaging, storage and alignment of images. A computer program integrates the 24 image stacks to produce a trace of each chromosome's position in the nucleus.

The first results, which were based on a single nucleus, revealed that the complexly-folded polytene chromosomes maintain a polarized orientation¹. That is, the chromocentre, a structure produced by fusion of all the centromeres, is situated at a fixed position on the nuclear membrane relative to the geography of the salivary gland. This position is opposite to that of the telomeres which also contact the membrane. The chromosomes are positioned close to the nuclear membrane, leaving the centre of the nucleus essentially free of chromosomes. These conclusions were similar to those previously obtained by less precise methods (see, for example, refs 2 and 3).

Technological improvements in image-

gathering and analysis have now enabled Sedat and co-workers to identify bands throughout the length of each chromosome arm and therefore to identify individual chromosomes⁴. Thus they have been able to determine whether specific cytological loci occupy similar spatial environments within each of six nuclei taken from the same salivary gland. Sedat's group has also introduced methods for describing spatial positions which are more objective and more easily quantified than those used in previous studies. To describe the intra-chromosomal folding patterns of chromosome arms, an intra-distance map is constructed in which the distance between a given cytological locus and every other locus on the chromosome arm is displayed⁴. In the map, the decrease in distance between two non-contiguous sites on a chromosome brought into proximity by folding is graphically illustrated as a point off the diagonal in the intra-distance plot.

The intra-distance maps obtained by Sedat's group for the same chromosome in different nuclei are not identical; for example, the X chromosome is not limited to a specific three-dimensional configuration. However, statistical analysis of folding patterns reveals that there are preferred configurations. Thus, in four of six nuclei, specific regions of the X chromosome are in apposition to each other. Despite extensive folding, individual chromosomes do not intertwine with other chromosomes but appear to occupy separate topological domains.

Inter-chromosomal distances were also measured. That is, it was determined how frequently specific regions on one chromosome arm are next to specific sites on a second. Preferred contacts between different chromosomes were detected but they are of a limited nature. For example, chromosome arm 2R often lies close to arm 2L but rarely near arm 3L. However, although 2L is found near 2R in all six

nuclei examined, the specific sites which are involved in these close contacts differ from nucleus to nucleus.

Finally, by determining the distance from each locus on each chromosome to the nuclear membrane, the specificity of membrane contact points was explored. Most of the chromosomes lie within 5 μm of the nuclear surface (nuclear diameter is 30–40 μm), but the position of many loci vis-à-vis the membrane varies considerably from nucleus to nucleus. Certain chromosomal sites are, however, tightly associated with the membrane in all or most nuclei. The sites correspond to a subset of those loci previously identified as sites of ectopic pairing (pairing between non-homologous chromosomes), late replication and susceptibility to breakage⁵. Note that specific membrane contact points, like intra-chromosomal folding patterns, though conserved, are not invariant features of interphase chromosomes.

Although genes were first viewed as discrete independent loci, it became apparent more than 50 years ago that the position of a gene can have a profound effect on its expression, lending credence to the hypothesis that the interphase nucleus is a highly ordered structure. However, since many chromosomal rearrangements are tolerated in *Drosophila*, nuclear architecture is likely to be flexible except, perhaps, for a limited subset of crucial loci. Sedat's approach has convincingly demonstrated that computer-assisted cytological studies can provide a wealth of positional information at the level of the individual genetic locus. The emerging picture is one of preferred, but not required, chromosomal configurations. To test the importance of these configurations, it will be crucial to determine whether the three-dimensional structure of polytene nuclei changes as a function of developmental stage or genetic makeup. □

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On 30 March 1984, the Marine Biological Association of the United Kingdom celebrates the centenary of its formation.

100 years ago

THE SOCIETY FOR THE BIOLOGICAL INVESTIGATION OF BRITISH COASTS

THE meeting held for the purpose of inaugurating a new society having the above title, took place last Monday in the rooms of the Royal Society, Prof. Huxley being in the chair.

Prof. Huxley began by observing that the establishment of marine biological stations had been undertaken during the last few years by most of the civilised countries, and was, indeed, a necessary result of the great change which had taken place in the aims of biological science. The study of development began about half a cen-

tury ago, and the ramifications of that inquiry, which had been extended to the mode of becoming of all live things by Mr. Darwin, had caused a complete change in the methods of biological research. In order to investigate the living being it was now no longer deemed sufficient, as in the days of our great-grandfathers, to observe its outside, or even in the days of our grandfathers, to examine its anatomy. We have now to trace its developmental growth from the egg, and we are able to do so with a thoroughness of which no one in his young days could have had any conception. Such was one good reason for founding an institution of this kind from a purely scientific point of view.

From *Nature* **29**, 533, 3 April 1884.

Astronomy

Classifying ourselves

from Virginia Trimble

DECIDING just how normal or average our star and Galaxy are is of practical as well as philosophical interest. The assumption that ours is a normal galaxy of a particular type is now being used in efforts¹ to pin down the extragalactic distance scale, the value of Hubble's constant and the age of the Universe, all still uncertain by about a factor of two. And properties of other stars are frequently determined by comparison with those of the Sun. Thus, if spectral type, colour, temperature and luminosity of the Sun are not known accurately, our ideas about how the chemical composition of the Galaxy has evolved with time, about the mass-luminosity relationship and even about the distance to the Hyades (which feeds back into the extragalactic distance scale) will come out wrong¹⁻⁴. Two recent papers^{5,6} represent the latest round of squabbles over whether the Milky Way is an Sb or an Sc spiral and whether the Sun is unusually red or normal for its spectral type.

Hubble⁷ and later workers^{8,9} classified spiral galaxies into two sequences, with central bars present (SB) or absent (S or SA). Both sequences are ordered by the degree of prominence of the spiral arms, from Sa, with bright nuclei and inconspicuous tightly-wound arms, through Sb to Sc, with very conspicuous loosely-wound arms. Many other properties of galaxies correlate well with Hubble type, including the zero points of the relationships (between total luminosity and rotational velocity, or surface brightness, or other distance-independent parameters) used to calibrate the extragalactic distance scale. The difference in luminosity between Sb and Sc galaxies at a given value of some other parameter is typically about a magnitude, corresponding to a 60 per cent error in distance if you guess the wrong type. As a result, we can only calibrate these relationships using data from our Galaxy if we know its Hubble type and that it is normal for its type with some precision. Unfortunately, we cannot photograph the Milky Way from outside and compare arms and nucleus optically. Thus all classifications of our Galaxy are more or less indirect and correspondingly uncertain. The advent of radio astronomy greatly increased the number of indirect indicators available¹⁰ and led Baade to decide that we live in an Sb galaxy¹¹. Later determinations⁵ have oscillated between Sb and Sc. The most detailed yielded the classification SAB(rs)bc¹², indicating intermediate status in all parameters, including existence of a ring, *r*.

If we accept this intermediate type, then our Galaxy is quite normal^{1,12-14}, having values of all the usual parameters

intermediate among those of four other galaxies of similar types. A composite drawing of the four shows more than two spiral arms, not all of equal brightness. And Hubble's constant, *H*, must be near 100 km s⁻¹ Mpc⁻¹ or the coherent picture falls apart. This last conclusion disturbs many astronomers, as it requires either that the Universe be less than 10¹⁰ years old or that Einstein's infamous cosmological constant be non-zero.

But suppose we are really an Sc. Galaxies of that type are intrinsically fainter than Sb galaxies. Thus when other galaxies are matched to ours, the effect is to make them intrinsically brighter, hence further away. According to Paul Hodge⁵, an equally coherent picture can then be assembled with *H* near 50 km s⁻¹ Mpc⁻¹, at least for luminosity and type determinations based on the scale length of the ionized hydrogen distribution in galactic discs. Classifications as Sc not depending on the distance scale¹⁵⁻¹⁷ then become arguments in favour of the lower value of *H*. It remains to be seen whether other distance-dependent indicators of Hubble type can also make the Milky Way look like a normal Sc with the larger distance scale. De Vaucouleurs (private communication) believes that this will not prove possible.

By contrast, the spectral type of our Sun cannot be debated. Modern systems of classification define G2V stars as those whose spectra are like the Sun's, at least at relatively low dispersion. During the 20 years from Kuiper¹⁸ to Stebbins and Kron¹⁹, there seems to have been general agreement that many other G2V stars indeed had absorption line spectra like the Sun's and were, on the average, the same colour, $(B-V) = 0.63$, and so at the same temperature.

Metal abundances for solar-type stars in the Hyades and other young clusters using these similarities turn out larger than solar by 20-50 per cent, implying that our Galaxy is becoming richer in heavy elements as generations of supernovae pour out synthesized nuclei. Most of us would like to believe all this.

Doubts began to gather only when modern photoelectric measurements of the solar colour²⁰⁻²² and indirect determinations, employing comparisons of spectral features with those of other stars²³, started yielding significantly redder colours of 0.65-0.69, typical of G5, but not G2, stars.

Hardop added to the difficulties by searching among the brighter stars for ones that would match the Sun in both broad-band energy distribution and detailed absorption line profiles^{2,3,6,24-26}. Such solar analogues are rare²⁶ — fewer than a dozen out of several thousand stars searched —

and nearly all are rather red, with $(B-V) = 0.65-0.71$. Most had been classified near G5, not G2; and three of the closest analogues are in the Hyades, implying that the Sun and these stars are rather similar in their abundances of the heavy elements.

What can have gone wrong? Chmielewski²⁷ has looked again at indirect measures of solar $(B-V)$, obtaining a best value of 0.633 ± 0.25 . This would permit the conventional wisdom to persist. He questions the accuracy of the direct measurements, all of which require some intermediary to bring small amounts of sunlight into a telescope and photometer that can also look at stars. But the most detailed direct determination yet²⁸ persists in finding 0.686 ± 0.01 , using three independent intermediaries.

Could spectral classification methods have unexpected bugs^{3,29}? Hardop has noted that the solar spectrum used as a standard is often that of sunlight reflected from Uranus or Neptune. Processes in the planetary atmospheres can partially fill in absorption lines, making the spectrum mimic that of a hotter star. Thus, when these artificially weakened lines are used as a standard, stars that really are like the Sun are classified as cooler, near G5. Once this is allowed for, then several of the solar-analogue stars end up as G2-3 (ref. 6), and $(B-V) = 0.67 \pm 0.01$ becomes normal for the type.

But since the Sun and its analogues in the Hyades then match in line profiles, detailed energy distributions and broad-band colours, they must have the same temperatures and the same compositions. If the galactic metal abundance has not changed in nearly 5,000 million years, then either supernovae have stopped expelling heavy elements (which sounds unlikely but is remarkably hard to disprove³⁰) or the products must be diluted by gas from

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regions where no massive stars have formed (less unlikely, but also hard to prove).

In summary, to conclude that the Milky Way is a normal Sbc galaxy and that the Sun is a normal G2V star, while philosophically satisfactory, leads to unexpected difficulties in understanding the scale of the Universe and the evolution of our Galaxy. This discussion may remind some readers of the revolutionary hypothesis advanced by Richard Armour³¹ that *Julius Caesar* was written neither at the end of Shakespeare's first period nor at the beginning of his second period (an ancient topic of controversy) but, rather, between two periods. If so, the blame attaches entirely to the present writer and not to those working on problems of stellar and galactic classification. □

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Astronomy

Latin-American gathering

from W.H. McCrea

THE latest in a series of regional meetings on astronomy, the third Reunion Regional Latinoamericana de Astronomia, in Buenos Aires, 28 November–3 December 1983, was yet another illustration of the value of these conferences, held under the general auspices of the International Astronomical Union. The meeting was attended by about 200 astronomers from at least six Latin-American countries as well as several invited speakers from North America and Europe. Although the general objective of the organizers is to hold regional conferences in this series at intervals of two years, the next meeting is to be late this year in Brazil.

The function of the meetings is twofold:

to give the communities of astronomers in the countries of Latin America a sense of belonging to a larger community; and to provide Latin-American astronomers, sometimes hindered by geography and problems of foreign exchange from attending the periodic general assemblies of their union, a sense of belonging to the international community.

In the past decade, Latin-American astronomy has been stimulated by the interest of astronomers from elsewhere who go to Chile to work in the new optical observatories in the Southern Hemisphere, as well as by a number of interesting bilateral collaborations with North American and European astronomers such as that between the Max Planck Institute for Radioastronomy (Bonn) and the Instituto Argentino de Radioastronomia on long-baseline observations.

On this occasion, Jorge Sahade (Argentina), a former vice-president of the union, was chairman of the scientific committee, which included Manuel Peimbert (Mexico), a vice-president of the union, and Victor Blanco (Chile), recently director of Cerro Tololo Inter-American Observatory.

The meeting showed that Latin America as a whole is active in most fields of astronomical research with current interest, both observational and theoretical. Clearly, this community has benefited from some access to the large internationally controlled telescopes in Chile and to some spaceborne facilities, the International Ultraviolet Explorer satellite for example. Many of the papers presented included co-authors from other parts of the world, but it seemed a pity that only a few of the astronomers from outside who make their observations in Chile were actually present in Buenos Aires.

In Argentina itself, astronomy has strong traditions in stellar astrophysics and in the studies of galaxies and galactic systems, centred particularly on the Córdoba and La Plata Observatories, as well as the radioastronomy institute. The observatories of Córdoba, La Plata and San Juan are to share in the use of a new 2.15-m optical reflector, the erection of which is nearing completion at El Leoncito in the approaches to the Andes. The dates of the conference had been chosen as near as practicable to the centenary of the founding of the La Plata Observatory in November 1883; some of the proceedings were designed to honour this occasion. Incidentally, this observatory has always included a strong geophysical section. □

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Shock waves from a bullet

SOME of the earliest photographs of the shock waves generated by a speeding bullet were taken in the laboratory of Ernst Mach at the German University of Prague in the winter of 1888. In 1884 Mach had tried to record such waves, using the schlieren technique and target pistols, but had failed because the muzzle velocity was subsonic.

At his behest, P. Salcher and S. Riegler at the Royal Imperial Naval Academy, in what is now Rijeka, Yugoslavia, experimented with larger projectiles and higher velocities and obtained successful photographs in 1886. In the same year, working with his son Ludwig on the artillery range at Meppen, Mach found that at any velocity v_p of the projectile greater than the speed of sound v_s in the medium, trailing shock waves formed a constant angle α with respect to the direction of motion regardless of the projectile's size, whence the equation: $\sin \alpha = v_s/v_p$.

When the Machs returned to the Physikalische Institute of the University of Prague,

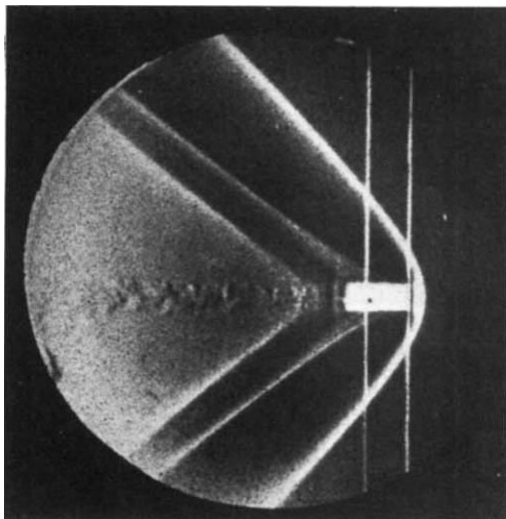
they gradually improved their apparatus in which a test bullet is illuminated by the brief spark discharge from a Leyden jar. In the photograph shown here, the bullet is a blunt cylinder of brass, 23 mm long and travelling at a speed of 520 m s⁻¹. The vertical white lines are copper wires, 0.5 mm thick, connected to opposite terminals. The bullet shorts the circuit. The original photograph, taken on Schleussner's extra-sensitive dry plates with an exposure of about 2 μ s, measures only 1 cm across.

The hyperboloidal leading wave looks merely hyperbolic in cross-section because the three-dimensional shell is too thin to reflect much light except when seen edge-on. Similarly, the trailing waves, which always point at a more rakish angle than the leading wave, are really cones seen in cross-section.

The Machs' experiments, and those by C.V. Boys the following decade, had repercussions beyond experimental physics. The fact that sharper bullets yield fewer eddies and lower drag, for example, was of considerable significance for artillerymen. Mach was quite aware of the military implications and once commented sardonically, "To shoot in the shortest time possible as many holes as possible in one another's bodies is the most important affair of modern life".

Jon Darius

One of the photographs in an exhibition 'Beyond Vision' at the Science Museum, London from 19 April. A book of the same title will be published in April by Oxford University Press. The photograph is reproduced by courtesy of the Ernst-Mach-Institut, Freiburg, FRG.



Lead cycling in estuaries, illustrated by the Gironde estuary, France

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The cycling of lead in estuaries involves a complex exchange between dissolved and particulate phases. In macrotidal estuaries such as the Gironde (France), dissolved lead is adsorbed onto particles due to an increase in turbidity and in the specific surface area of particles in the tidal estuary. In the lower estuary a mobilization of exchangeable and carbonate lead occurs simultaneously with a coagulation of organic dissolved lead associated with iron-manganese hydrous oxides. The lead-depleted particles are subsequently recycled to the tidal estuary by landward currents, where they become mixed with fresh river-borne particles. Lead isotopic ratios and particulate lead speciation studies indicate that in this estuary the mixing of polluted particles with old deposits probably has a minor role in the decrease in particulate lead.

As river water runs into the ocean, it is in effect entering a reservoir where it can accumulate up to the age limit of the reservoir itself, unless its concentration is controlled at some intermediate level by removal processes (generally through interactions with particulate matter)^{1,2}. The behaviour of dissolved chemical elements can be roughly predicted from the relative residence time of that element in the ocean which in turn depends on the ratio between the ocean and river water concentrations. For many trace metals such as iron and manganese there is a net removal of dissolved species during estuarine mixing^{3,4}. As far as the particulate phase is concerned, more than 90% of sediments and associated trace metals supplied by the rivers settle in estuaries and nearshore areas.

Such global approaches allow us to predict that most of the dissolved and particulate lead supplied by rivers will be deposited in the estuarine and coastal zones. However, this general picture is more relevant to the long-term control of seawater composition and the geological history of ocean sedimentation than to the thorough understanding of Pb cycling at a regional level and over a short time scale.

In this article, the origin, concentration and fate of Pb have been investigated in a representative macrotidal estuary, that of the Gironde river in France. Most previous studies of this estuary considered the particulate phase and assumed that a significant mobilization process occurred as soon as the river water mixed with the sea water^{5,6}. Duinker and co-workers, demonstrated clearly the predominance of mixing over desorption processes⁷. Particulate and dissolved concentrations (total concentrations and specific forms) and isotopic composition have been measured to resolve these problems.

Sampling and analysis

The Gironde estuary is located in south-west France (Fig. 1), and is formed by the confluence of the Garonne and Dordogne rivers. Mean annual discharge is $24 \times 10^9 \text{ m}^3 \text{ yr}^{-1}$ and solid discharge $2.2 \times 10^{12} \text{ g yr}^{-1}$, two-thirds of which is supplied by the Garonne river, which represents the main tributary. The Gironde can be considered as a partially mixed estuary characterized by a relatively long flushing time (ranging in average tidal conditions from 20 days in flood to 86 days during the low water stage). Hydrodynamic mechanisms in the Gironde estuary produce a well developed 'turbidity maximum' ranging from 0.1 to 10 g l^{-1} . Moreover, during certain periods a 'fluid mud' layer, where turbidity reaches 400 g l^{-1} , accumulates on the estuary bottom, particularly along the navigation channel. The total sediment mass in these two turbidity accumulations (5×10^6 tonnes) is twice the annual river solid discharge, corresponding

to an estimated 'residence time' of 2 months to 2 yr. This lengthy time spent by the water and particulate matter within the estuarine system as well as the large numbers of particles will greatly favour solid-liquid interactions. The estuary is divided into two parts: the middle or saline estuary, corresponding to the mixing zone between river and sea waters, and the upper or tidal estuary characterized by fresh water but still subjected to daily tidal intrusion.

Four sampling surveys were carried out. For these, water samples were collected in polyethylene bottles which had been soaked for 3 days in hot HCl 30% analytical grade, rinsed with demineralized water, then transferred to a clean-air cupboard and kept filled with HCl 5% (Merck Suprapur) for 7 days. The bottles were rinsed three times with demineralized water and transferred to polyethylene bags. Before each sampling the bottles were rinsed with sample water to condition them. Demineralized water acidified to pH 2 over 3 months in these precleaned bottles showed no contamination. The water samples were filtered under nitrogen pressure on $0.4 \mu\text{m}$ acid-cleaned Nuclepore filters. All manipulations were performed in a laminar airflow cupboard. The filtrate was acidified to 1% with HCl (Merck Suprapur), and the filters were oven dried for 24 h at 105°C . The particulate matter was recovered from the filters in a clean-air cupboard and subsequently ground in an agate mortar.

Total Pb concentrations in suspended matter were determined by atomic absorption spectroscopy (IL 453) after digestion with a mixture of HNO_3 , HClO_4 and HF (Merck Suprapur). Blanks were negligible. The same digestion was used for Pb isotopic analysis. The Pb separation and ultrapurification were achieved by anion exchange techniques⁸ and the isotopic ratios measured on a VG Micromass 5438. The total Pb blank was 20 ng.

Particulate Pb speciation, determined according to Tessier *et al.*⁹, revealed five fractions: exchangeable, carbonate, Fe-Mn oxides, organic and residual fractions. Particulate scandium was determined on an aliquot using INAA.

Total dissolved Pb was measured by spiking a 50-cm^3 HCl-acidified aliquot (pH 2) with 1 ml of HNO_3 and UV irradiating for 12 h. The UV irradiation device used was that described previously by Mart *et al.*¹⁰ to photooxidize dissolved organic components that might bind Pb species inaccessible to DPASV. After UV irradiation the pH of the sample was readjusted to 1.5 by adding 1.2 ml of NH_4OH and the voltammetric determination was performed according to Mart *et al.*¹⁰, using a PAR 174 (Princeton Applied Research) equipped with a rotating glassy carbon electrode (provided by L. Mart) or a Metrohm glassy carbon electrode.

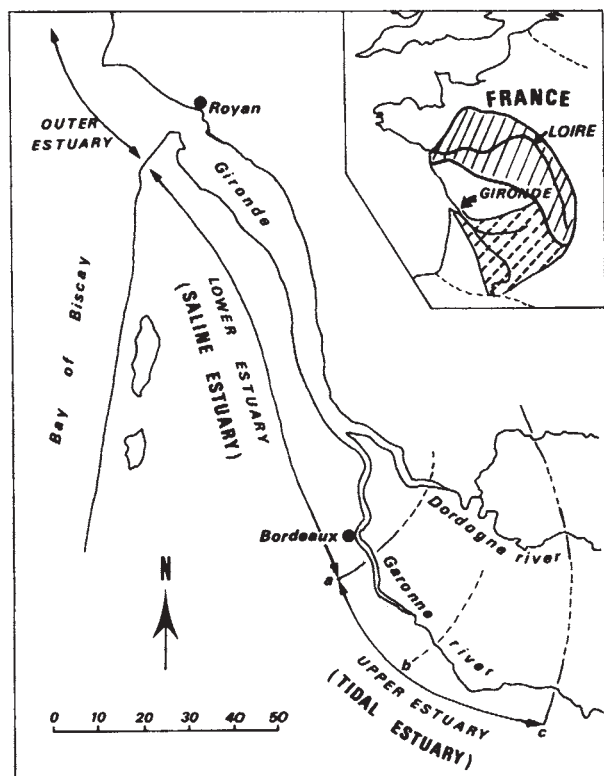


Fig. 1 Sampling stations for dissolved lead in the Gironde estuary. a, Upper limit of salt intrusion; b, approximate upper limit of tidal node; c, upper limit of tides.

'Organic' dissolved Pb was calculated as the difference between total Pb (determined after UV irradiation and at pH <2) and free and labile lead measured at pH 2 before UV irradiation. The result obtained for organic Pb represents a minimum value because the acidification of the water sample during storage may have destroyed the more labile organic Pb species.

Contamination due to filtration has been estimated to be ≤ 1 ng per kg, while acidification of the samples introduced a maximum contamination of 2.5 ng per kg. Finally, the addition of the various reagents needed for analysis (20 μ l HCl, 25 μ l $\text{Hg}(\text{NO}_3)_2$, 100 μ l KCl) corresponded to 3 ng per kg contamination. For the determination of total Pb, subsequent addition of 1 ml HNO_3 , 1.2 ml NH_4OH and UV irradiation added a Pb contamination of 2 ng per kg. The total blank then reached a maximum value of 7.5 ng per kg, well within the river-estuarine concentrations encountered in the Gironde (<10%).

Absolute standard deviation ($\pm 2\sigma$), tested by making replicate determinations on seven river samples, varied from 0.8 for the range of concentrations observed in the Gironde estuary (60 ng per kg) to 5.7 ng per kg for higher concentrations. Accuracy was tested by analysing the NASS-1 (National Research Council Canada) seawater reference sample. The results obtained agreed well with the recommended value (39 ± 6 ng per kg).

Total Pb

The results obtained in various hydrological conditions were compared by plotting the data as a function of chlorinity (x-axis) in the saline estuary and as a function of the distance to the river reference point for the samples located upstream of salt intrusion (tidal estuary).

Pb concentrations in suspended matter (Fig. 2a) were very similar for March 1978 and October 1979 samples. The average river concentration is $70 \mu\text{g g}^{-1}$, decreasing to $60 \mu\text{g g}^{-1}$ in the tidal estuary. To correct for possible variations due to grain

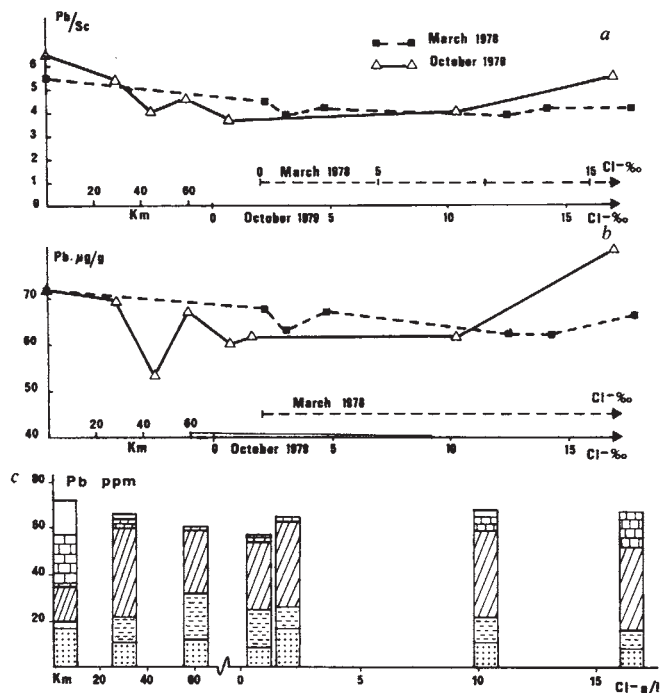


Fig. 2 Particulate lead in the Gironde estuary from March 1978 (■) and October 1979 (△): a, without normalization; b, after Sc normalization; c, particulate lead speciation in the Gironde estuary suspensions (October 1979) according to the sequential procedure of Tessier *et al.*⁹. □, Exchangeable fraction, 1 M MgCl_2 (pH 7.0); ▨, carbonate fraction, 1 M NaOAc (pH = 5.0 HOAc); ▩, Fe-Mn oxide fraction, 0.04 M NM_2OH , HCl (pH 2, 25% HOAc); ▤, organic fraction, 30% H_2O_2 (pH 2 HNO_3), 3.2 M NH_4OAc (20% NHO_3), 3.2 M NH_4OAc (20% NHO_3); ▥, residual fraction HF, HClO_4 .

size, the absolute concentrations were normalized to scandium, which resulted in a greater relative decrease (30%) (see Fig. 2b).

Dissolved total concentrations in the rivers ranged from 184 ng per kg to 152 (Garonne) and 95 (Dordogne) ng per kg (Fig. 3, Table 1). A general decrease in dissolved Pb concentrations from the river to the salt intrusion was observed. These values are higher than those measured in the Mississippi River and in rivers flowing to the South Atlantic Bight in USA (H. Windom, personal communication), or in Italian rivers¹¹ and other French rivers such as the Rhône, where the river Pb concentration is 70 ng per kg despite the high level of industrialization (F.E.-P., unpublished). The higher concentrations in the Garonne and Dordogne rivers are probably due to the presence of Pb ores, especially in the Garonne drainage basin. In the lower estuary, higher concentrations of dissolved Pb were measured in 1981 samples whereas dissolved Pb concentrations were fairly constant for 1982 samples. In 1981, some sampling stations were located near sources of contamination: the oil refinery at Pauillac, and the Verdon Harbour. In our assessment of Pb geochemistry these contaminated areas were carefully excluded from the 1982 survey; sampling stations were located far from harbour areas and industrial discharges. Note, however, that these dissolved concentrations are still higher than those found on the inner shelf about 20 miles from the mouths of the Gironde and Loire rivers (F.E.-P., unpublished); these average 40 ng per kg, a concentration which has recently been confirmed by an intercalibration study performed by the ICES.

Pb speciation

In river water, the range of organic dissolved Pb is 55–80% of total dissolved Pb (Fig. 3), whereas in the Gironde estuary it represents only 20–30%. Interestingly, the decrease in organic dissolved Pb occurs before the saline intrusion. With regard to free/labile dissolved Pb, from the river to the sea, there is a

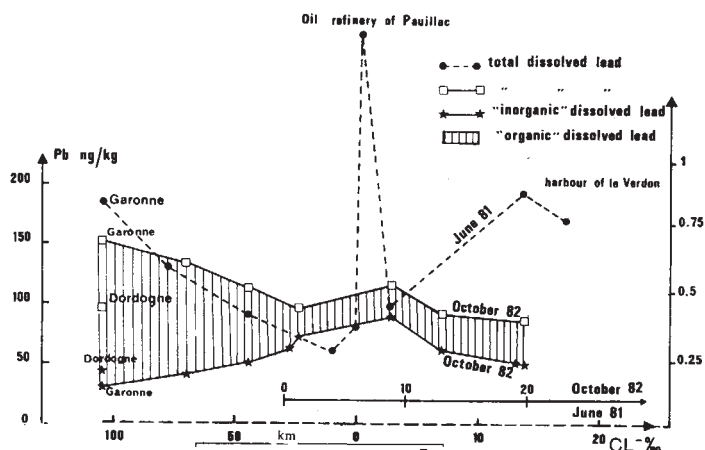


Fig. 3 Dissolved Pb in the Garonne and Dordogne rivers and in the Gironde estuary (June 1981, high water stage; October 1982, low water stage).

correlative increase in both its concentration and percentage, with a slight maximum in the estuary.

Particulate Pb speciation studies showed that, from the river to the sea, the carbonate-bound and exchangeable Pb decreased while bound Fe-Mn oxides and organic Pb increased. The proportion of Pb present in the various fractions was recalculated as a percentage of total Pb concentration measured in the untreated sample (Fig. 2c). The Pb content of the carbonate fraction decreased from 31% to a negligible amount downstream and increased again to 9–26% near the river mouth. The Pb associated with the exchangeable fraction decreased from 21% upstream to a negligible amount in estuarine samples, while the Pb in the Fe-Mn oxide fraction increased from 21% upstream to 61% in estuarine samples, and the Pb in the organic fraction increased from 4% in the river samples to 33% for the sample taken just upstream of the salt intrusion.

Mixing with marine particles

As indicated above, a slight decrease in total particulate Pb was observed in the tidal estuary. This decrease could be explained by a mixing of high river Pb concentrations with estuarine particles characterized by a lower content of trace metals. Several possible reasons for this are discussed below. Altern-

Table 1 Dissolved total lead and free/labile lead in the Gironde estuary (June 1981–October 1982)

Sample	Cl%	Dissolved Pb (ng per kg)	
		Free/labile	Total
Gironde 1981			
G 8101 (Garonne)	0.008		184
G 8102	0.008		134
G 8103	—		90
G 8109	0.010		60
G 8119	0.017		80
G 8114	0.400		327
G 8115	2.560		98
G 8120	13.61		194
G 8121	17.19		170
Gironde 1982			
G 8210 (Dordogne)	0.017	43	96
G 8209 (Garonne)	0.018	30	152
G 8208	0.024	41	134
G 8207	0.021	51	113
G 8205	1.22	72	97
G 8204	8.76	91	115
G 8203	13.56	62	92
G 8201	19.75	60	87
Inner shelf	19.9	—	47

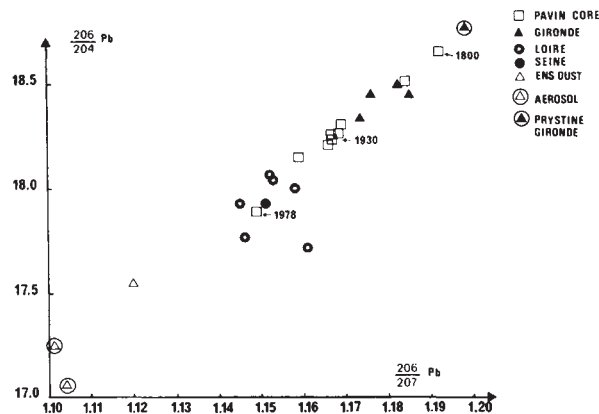


Fig. 4 Lead isotopic composition of particles: □, Pavin crater lake (Massif Central, France) cored sediments; ▲, Garonne river and Gironde estuary suspensions (March 1978); ●, unpolluted Gironde; ●, Seine (1979) and Loire (April 1979–June 1982) estuary suspensions; △, laboratory dust collected at ENS, Paris; ⊙, filtered aerosols sampled at a car park near Paris in 1982.

tively, the decrease could be attributed to a mixing of river suspensions with marine suspensions. Indeed, coarse marine sands originating from the inlet can be transported 15–25 km up the estuary due to the residual landward bottom currents¹². For fine-grained sediments in the river and upper estuary, $\delta^{13}\text{C}$ values are typical of those for continental organic matter (–24, –26). A slight increase (from –15 to –20) was observed in the saline part of the estuary, near the mouth, but no characteristic marine organic matter ratios were found in the upper estuary itself¹³. These results indicate that most of the estuary is under the influence of terrigenous sediments and that penetration of marine material is limited to the lower reaches of the estuary. On the other hand, the concentrations of trace metals in the suspensions sampled near the mouth are generally slightly higher than in the turbid zones of the middle estuary. On a larger scale, it is well known that suspensions from the open ocean are enriched in most trace metals as compared with the Earth's crust¹⁴. These sedimentological and geochemical data suggest that the lower Pb concentrations observed in suspensions from the Gironde estuary cannot be accounted for by their dilution with marine particulates.

Origin of Pb

The decreased estuarine particulate Pb concentration could result from the mixing of contaminated river suspensions with non-contaminated 'preindustrial' estuarine deposits. Recycling of such old sediments could occur either by various natural processes (for example, bank erosion, reworking due to channel migration, tidal currents) or through human activities (such as dredging operations). To clarify this problem, we attempted to determine the origin of the Pb by its isotopic composition.

It is well known that most of the Pb found in the environment over the past 10 years comes from alkyl Pb compounds added to petrol for their antiknock properties. The isotopic composition of the present atmospheric Pb is thus restricted to the range of major Pb ores used in the manufacture of Pb alkyls. Chow has shown that, for economic and political reasons, the lead in petrol originates from a limited number of mineral ores¹⁵. Several studies have demonstrated the feasibility of using the Pb isotopic composition to trace the source of Pb pollution in different environments (glaciers, sediments, estuaries), particularly in ponds (refs 16, 17 and F.E.-P. *et al.*, in preparation) and estuaries¹⁸.

In France, where Octel is the only supplier of alkyl Pb, the $^{206}\text{Pb}/^{207}\text{Pb}$ ratio in petrol from Paris in 1966 was 1.162 (ref. 19) (for clarity only this ratio will be discussed), and had decreased to 1.128 in grass sampled near a highway in Paris in 1975 (D. Petit, personal communication). Recent studies on aerosol samples collected near Paris give a value close to 1.10. This makes it easier to differentiate between recent

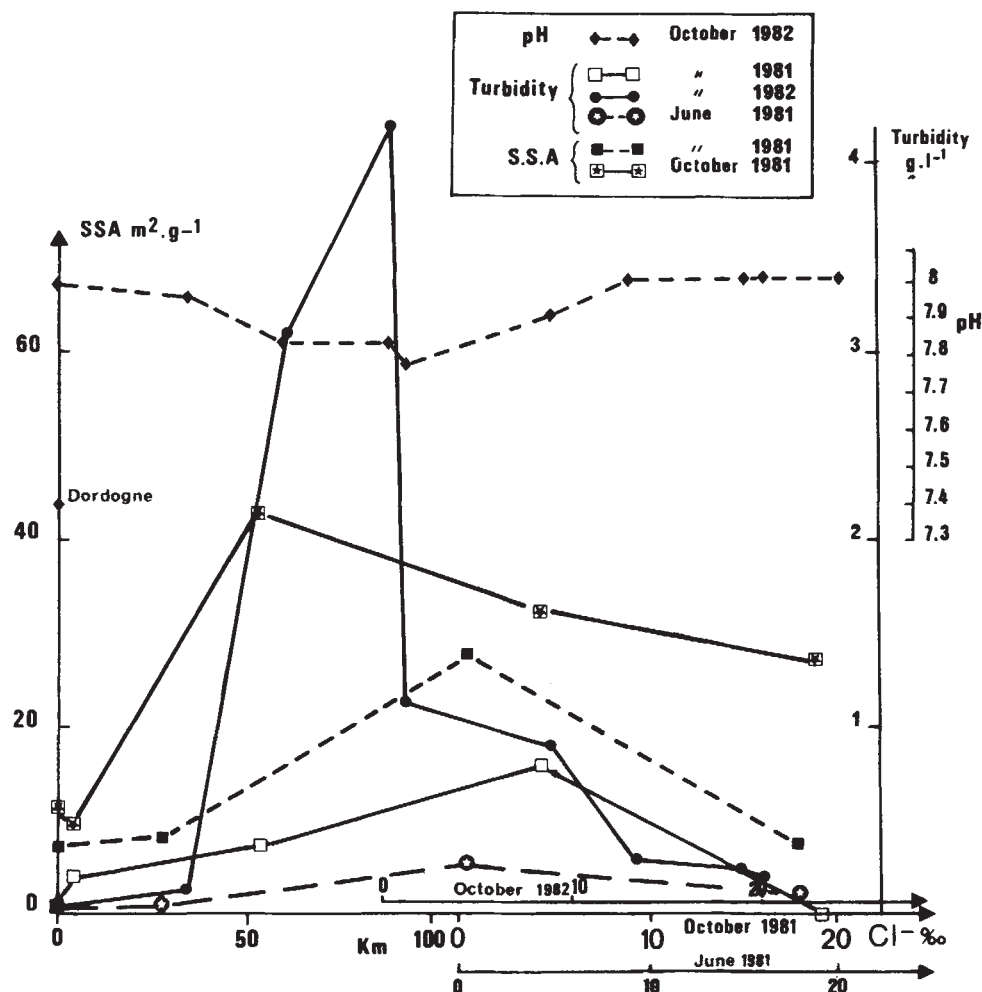


Fig. 5 Turbidity (●, June 1981; □, October 1981; ●, October 1982) and pH (◆, October 1982) in the Gironde estuary, and surface specific area in the Garonne river and Gironde estuary (■, June 1981; ☆, October 1981).

anthropogenic Pb and natural Pb, for which an isotopic ratio of 1.18 was found in preindustrial lacustrine deposits of the Pavin crater lake located very close to the drainage basin of the Dordogne and Garonne rivers (F.E.-P. *et al.*, in preparation). A slightly higher value (1.184) has been measured in an ancient deposit collected near Bordeaux which can be considered as representative of the Garonne sediment supply 5,000–7,000 yr ago.

The $^{206}\text{Pb}/^{207}\text{Pb}$ ratio (Table 2, Fig. 4) (1.173 in Garonne suspensions and 1.179 in the Gironde estuary) corresponds to an important proportion of natural Pb. The slightly higher values encountered in the lower estuary have recently been confirmed by a detailed study of Pb isotopes in French estuaries (F.E.-P., P.H., J.-M.M. and D. Petit, in preparation). These more radiogenic ratios imply a higher percentage of natural Pb in the lower estuary than in the river. For comparison we have also measured Pb isotopic ratios in two other major French estuaries (Loire and Seine, see Fig. 4), and have found a $^{206}\text{Pb}/^{207}\text{Pb}$ value close to 1.15, identical to that measured in recent Pavin lacustrine deposits, and thus indicating a predominant anthropogenic component for Pb in suspended matter from these two latter estuarine systems (Table 2). The slight increase in the estuarine isotopic ratio can be ascribed to a mixing with uncontaminated preindustrial sediments, but as shown by artificial radionuclide data⁶, the 'age' of the particles ranges from 2 months to 2 yr, which makes this hypothesis unrealistic. Another possibility would be the preferential mobilization of the anthropogenic Pb fraction.

Pb mobilization

Such mobilization processes from the particulate to the dissolved phase have been clarified using particulate Pb speciation data;

however there is no definite evidence as to where these processes occur, in the water column itself or in the temporarily deposited sediments. The decrease in total Pb content in the particulate matter is associated with a similar trend for Pb associated with carbonates and Pb in the exchangeable fraction.

With regard to the water column, the decrease in carbonate-associated Pb reflects the carbonate content of the suspensions, and might be explained by partial dissolution of calcite due to a pH decrease from 7.91–8.1 to 7.5–7.8 in the estuary (Fig. 5). The decrease of Pb in the exchangeable fraction could be attributed to competition between major seawater cations (Mg^{2+} , Ca^{2+} and Pb^{2+}) for the adsorption sites on suspended matter. Another explanation is the slight decrease in the excess charge at the surface of suspended matter due to the combined effect of the decrease in pH and increase in ionic strength during estuarine mixing⁶.

With regard to the temporarily deposited sediments, a pH decrease has also been observed in pore water²⁰, which could lead to a dissolution of Pb associated with carbonates. A part of this dissolved Pb could diffuse to the overlying water or precipitate as PbS , resulting in the increase of Pb observed in the so-called organic fraction which also contains sulphur-associated Pb. Further, the decrease in the oxidation–reduction potential of the sediment leads to a solubilization of Pb associated with Fe–Mn oxides; part of it precipitates immediately, the other part diffuses to the overlying water.

These desorption processes might occur in the 'saline' estuary where the particles become depleted of Pb and are subsequently transported landwards to the 'upper estuary' where they mix with fresh river particles. Such a mechanism can explain the particulate Pb decrease in the Gironde estuary. It is surprising that no excess total dissolved Pb is observed. One must remem-

Table 2 Particulate isotopic composition and particulate lead concentrations in various river and air samples

Sample	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{207}\text{Pb}/^{204}\text{Pb}$	$^{208}\text{Pb}/^{204}\text{Pb}$	$^{206}\text{Pb}/^{207}\text{Pb}$	$\mu\text{g per g Pb}$
Loire					
ICOLO 26.3	17.955 ± 0.012	15.505 ± 0.010	37.660 ± 0.025	1.1580 ± 0.0004	—
ICOLO 26.2 (1)	17.930 ± 0.060	15.660 ± 0.040	37.80 ± 0.1	1.1448 ± 0.0007	—
ICOLO 26.2 (2)	17.774 ± 0.019	15.503 ± 0.021	37.473 ± 0.058	1.1464 ± 0.0002	—
ICOLO 26.1	17.720 ± 0.011	15.248 ± 0.013	36.842 ± 0.037	1.1624 ± 0.0004	—
L79 P01	18.06 ± 0.04	15.65 ± 0.02	38.06 ± 0.10	1.1519 ± 0.0015	87
L79 G50	18.04 ± 0.04	15.64 ± 0.03	37.92 ± 0.10	1.1527 ± 0.0016	108
Seine					
S79 Elboeuf	17.93 ± 0.03	15.58 ± 0.02	37.85 ± 0.10	1.1514 ± 0.0015	169
Gironde					
G 7801	18.34 ± 0.03	15.63 ± 0.02	38.10 ± 0.05	1.1734 ± 0.0008	72
G 7806	18.48 ± 0.05	15.68 ± 0.05	38.50 ± 0.20	1.177 ± 0.003	67
G 7810	18.45 ± 0.05	15.57 ± 0.04	38.35 ± 0.15	1.185 ± 0.003	60
G 7821	18.47 ± 0.04	15.65 ± 0.04	38.45 ± 0.20	1.177 ± 0.002	62
Flandrian unpolluted Gironde	18.753 ± 0.002	15.649 ± 0.002	38.721 ± 0.006	1.19841 ± 0.00007	33
Pavin					
Upper level	17.887 ± 0.010	15.571 ± 0.007	37.784 ± 0.024	1.14874 ± 0.00018	70
Lower level	18.512 ± 0.014	15.629 ± 0.012	38.523 ± 0.031	1.18446 ± 0.00027	9
Air					
Paris suburb					
December 81	17.25 ± 0.05	15.56 ± 0.05	37.49 ± 0.13	1.101 ± 0.001	—
ENS dust					
1	15.55 ± 0.04	15.66 ± 0.03	37.65 ± 0.05	1.120 ± 0.001	1,000
2	—	—	—	1.150 ± 0.010	—
NBS standard SRM-981	16.903 ± 0.003	15.445 ± 0.003	36.54 ± 0.02	—	—

Pavin crater lake (Massif Central, France) cored sediments; river, Gironde estuary suspensions (March 1978); Seine (1979) and Loire (April 1979–June 1982) estuary suspensions; laboratory dust collected at the European Nuclear Society (ENS), Paris; filtered aerosols sampled at a car park near Paris in 1982; Garonne, unpolluted Gironde.

ber, however, that the decrease in particulate trace metal cannot be related to a corresponding excess in the dissolved phase because of the different residence time of sediment and water, and because the dilution of estuarine water by the tidal seawater influx is 30–500 times greater than the river discharge at the inlet.

Pb removal

Conversely, there is a striking decrease in total dissolved Pb in the upper estuary before the saline intrusion. This decrease is associated with a similar trend for organic dissolved Pb and could be due to selective adsorption onto particulate matter: organic dissolved Pb could be either adsorbed directly onto particulate matter and/or transformed into free/labile Pb. This transformation would be followed by the adsorption of part of the corresponding free/labile Pb onto particulate matter. As Fig. 5 shows, the turbidity and the specific surface area (SSA) of the particles increase in the tidal zone, leading to an increase in the number of adsorption sites and favouring the adsorption of Pb, which has a very high distribution coefficient (K_D , 4.7×10^4 – 9.1×10^5) in the Gironde estuary. Indeed, more than 90% of Pb occurs in the particulate phase.

The observed decrease in organic dissolved Pb from the river to the sea is not surprising. As Mantoura *et al.* have shown²¹, more than 99% of the humic material is complexed by Ca and Mg in seawater. Moreover, a decrease in dissolved organic carbon (DOC) has been observed from the river to the sea. In certain hydrological conditions (March 1978, June and October 1981) the average river DOC is 2.2–3.5 mg l⁻¹ and decreases to 0.9 ± 0.3 mg l⁻¹ at the river mouth.

We have not observed any increase in total particulate Pb because any adsorption is masked by the desorption from the carbonate and exchangeable fractions, but we have observed an increase of Pb in Fe–Mn oxide and organic fractions. Other studies^{4,6,22} have indicated an increase in the Fe–Mn oxide phase due to coagulation of Fe and Mn as soon as the salinity becomes significant. This process facilitates the incorporation of Pb into this fraction of the particles when they are transported upstream. The phenomenon is accentuated by the fact that, together with the carbonate fraction, the Fe–Mn fraction is the most Pb-reactive particulate phase²³.

Pb cycling

The observed decrease in particulate Pb from the river to the estuarine zone in the Gironde estuary cannot be explained by mixing processes of polluted river particles with preindustrial estuarine particles. A mobilization process is needed to explain particulate Pb distribution. Several physicochemical processes, for example carbonate dissolution, competition with major seawater cations and decrease in the excess electrical charge at the particle surface, can explain the decrease. This mobilization occurs within the lower estuary either in the water column or in the temporarily deposited sediments where the particles are depleted in Pb and subsequently transported above the salt intrusion where dissolved Pb can be adsorbed onto particles due to increased turbidity and SSA (adsorption zone) (Fig. 5). Meanwhile, the mobilization processes are counterbalanced by Fe–Mn coagulation and Pb precipitation or coprecipitation.

A similar complex behaviour involving both mobilization and removal processes has been observed by Morris *et al.* for Mn in the Tamar estuary²⁴. In the case of the Gironde estuary, which is characterized by a long residence time for water and particles, these heterogeneous reactions become predominant. However, they can be overwhelmed by sediment transport and recycling due to physical processes.

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Characteristic folding pattern of polytene chromosomes in *Drosophila* salivary gland nuclei

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A computer-based system for recording and analysing light microscope images, combined with classical cytogenetic analysis, has revealed the spatial organization of the giant chromosomes of Drosophila salivary gland cells. Each polytene chromosome arm folds up in a characteristic way, contacts the nuclear surface at specific sites and is topologically isolated from all other arms.

THERE is little direct observational evidence for a large-scale regular architecture in interphase nuclei (briefly reviewed in ref. 1). Extensive indirect evidence for a determined structure comes from studies of transvection^{2–4}, position-effect variegation^{5,6}, chromosome rearrangements^{7–10}, and ectopic pairing^{11,12}.

We have chosen to work with the polytene chromosomes of the *Drosophila* salivary gland for several reasons: (1) they are large, having gone through about 10 rounds of replication without nuclear division, throughout which the homologous chromatids have remained in almost perfect lateral alignment, thus rendering the interphase chromosomes visible under the light microscope; (2) there are only five major arms (X, 2L, 2R, 3L and 3R); and (3) they have a highly determinate pattern of ~5,000 condensed chromatin regions, known as bands, which have been catalogued in detail^{13,14} and which serve as a unique physical reference for correlation of structure to an extensive body of genetic data. By analysing nuclei in the paired glands of a single larva, variations due to differences in genetic constitution, developmental state and sex are minimized.

An initial analysis of one polytene nucleus was reported recently¹. The present work incorporates several improvements over the previous study. The image recording hardware has been upgraded: (1) Images from the computer-controlled microscope are now digitized directly from the video camera instead of from photographic negatives, thus avoiding alignment errors. (2) Each recorded image contains 512×512 pixels instead of the previous 128×128; the resolution has thus been improved to the point where the chromosomal banding patterns can be read, and this allows us to make meaningful comparisons between nuclei (see Fig. 1). (3) The tracing of the chromosome pathways is now accomplished with an interactive computer modelling program, supplanting the half-silvered mirror and plastic sheets used before in a Richards' box. In short, the input data are of much finer detail, and the analysis is both more rapid and more objective. The structural features elucidated in the present investigation are consistent with the description of the overall morphology given in our previous study¹.

We report here the analysis of a set of nuclei in the unfixed salivary glands of a *Drosophila melanogaster* larva. The appearance of striking organizational motifs based on a quantitative comparisons of the paths of the chromosomes implies the existence of an underlying order is present beneath the considerable flexibility in the overall structure.

Data collection and analysis

The optical arrangement and computer hardware and software are described in detail elsewhere^{1,15,16}, as is the preparation of the salivary glands for microscopy^{17,18}. Briefly, salivary glands from late third-instar *D. melanogaster* larvae (Oregon-R, inbred for 38 generations) were carefully dissected into a buffer optimized for preservation of chromosome structure^{17,19}, stained with the DNA-specific fluorescent dye 4',6-diamidino-2-phenylindole (DAPI), and mounted on an inverted, computer-controlled Axiomat microscope. By stepping the microscope focus through each nucleus in small increments, successive planes of the sample were brought into view (Figs 1, 2). These images were digitized and stored in the computer. A stack of 24 such images was taken for each nucleus. Using our Interactive Modelling Program, IMP¹⁵, the paths of the different chromosome arms were traced out (see legends to Figs 1 and 2). These traces constitute the model (Fig. 3) on which all subsequent computations were based. Models were built for 14 of the nuclei, 6 of which were analysed in detail. Figure 2 indicates the overall scheme for the data collection and transformations, and also points out some of the advantages of these novel techniques.

The central question addressed in this study was whether the configurations of the chromosomes within an interphase nucleus are in some way determined. If so, one expects that in a single tissue at a given time in development, there will be similarities between these configurations in all the nuclei. To compare accurately the models derived from these nuclei, a set of parameters was defined which describes various orientation-independent properties of the paths traced along the chromosomes. These were plotted in a series of graphs as a function of cytological position along the chromosome (for example, see Fig. 6).

Plots are presented as a function of cytological position, rather than linear position along the chromosome, because of certain

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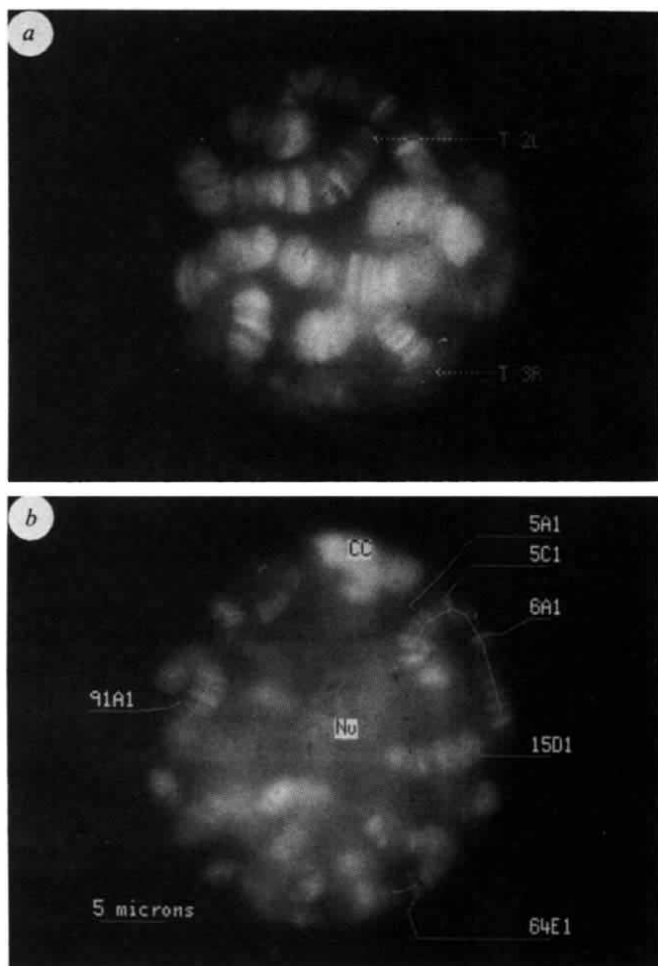


Fig. 1 Two sample frames from the original data sets. Each represents the averaged digital image (512×512 picture elements or pixels) of 256 video frames. Twenty-four such images, comprising 24 successive focus steps through a nucleus, make up the image stack, and take about 4 min to collect. Each of the approximately 6 million pixels in the stack has an intensity value of 0–255 associated with it. The chromosome banding patterns visible in these preparations are virtually the same as those seen in unstained squashes. It is possible to roughly stage this gland at about PS7 or PS8 using the well established puffing sequence which characterizes the late third-instar/prepupal stages of development⁴⁰. *a*, A frame near the top of an image stack from a nucleus. At least partially visible in this plane are two telomeres (T). *b*, A frame in the middle of a stack from another nucleus: the chromocentre (CC) and part of the nucleolus (Nu) are visible. Some cytogenetic loci are indicated. Lines along the chromosomes indicate the model paths.

Methods: Salivary glands from late third-instar larvae were dissected into modified buffer A (60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 15 mM PIPES buffer, pH 7.5)¹⁹ then stained in the same buffer with 100 μ g/ml DAPI (Sigma) for 1 h. After washing, glands were mounted between bridged coverslips sealed with paraffin oil. Samples were placed on a modified Zeiss Axiomat inverted microscope. Epifluorescence optics included a $\times 100/1.3$ n.a. Planapo objective, a 420 nm dichroic mirror, and a 425 nm barrier filter. Excitation was by the 405 nm Hg emission line. Images were collected by a DAGE-MTI model 66 SIT camera and passed to an RCA TC1254 monitor as well as to the Gould/DeAnza IP6400 image processor. The focus step size was adjusted so that the entire nucleus would be contained in 24 successive steps. A VAX 11/780 minicomputer controlled the focus through a Commodore PET microcomputer. Stepping of the focus, image averaging, and storage of images in the computer were all handled by IMGEDT, a general purpose image processing program¹⁵. The IP6400, equipped with a Barco CD51HR colour monitor, was also used in the subsequent model constructions and analysis. These and all subsequent photographs of data were taken on a Dunn Instruments 632 Colour Camera System.

inherent complications in the use of the latter. The length of the same arm from different nuclei is not necessarily the same, for several reasons. The proximal endpoints of the arms are not well defined because the chromosomes are difficult to follow in the large, intensely fluorescing region where all the centromeres fuse (the chromocentre). Differences in the compression within the arms themselves and small modelling errors also lead to variation. To circumvent this problem a set of fiducial marks along the arms is needed which can be used to align the chromosomes from different nuclei. Conveniently, these are provided in the polytene banding patterns, for which detailed cytological maps exist^{13,14}. With these maps as a guide, the positions of specific chromosomal regions within the intact nuclei could be determined (see Fig. 2 legend). Also, to facilitate comparisons of quantities plotted as a function of cytological position, these marks (bands) were fitted onto a standard grid with intervening points placed by linear interpolation (Figs 4–6). An important additional benefit of overlaying the cytology on the chromosome paths is that it serves as a check on the correctness of the models.

Visual inspection of nuclear models

Several significant results can be gleaned from the models even without knowledge of the detailed cytology. The most obvious is that the chromocentre always abuts the nuclear envelope (Fig. 1*b*). This is in agreement with what is seen in *Drosophila* early embryos²⁰ and also in other organisms^{10,21}. If this structure were randomly placed in the nucleus, one would expect to see it located internally in some of the nuclei, but this is never the case. Whether or not it can slide along the nuclear surface cannot be determined from these data. There do seem to be limits on the extent to which it may roam this interior surface; in all the nuclei investigated, this structure is always in the outer hemisphere of the nucleus, that is, away from the lumen of the gland and towards the exterior. Interestingly, centromeres are polarized towards the outside of the embryo²⁰. A large percentage of each chromosome also runs along the envelope's inner surface. In most nuclei a large fraction of each chromosome is curled into a series of loops or irregular helices with the telomeres lying at the opposite side of the nucleus from the chromocentre (Fig. 3*b*). In all nuclei where these rough helices are apparent for several turns, they are wound in a right-handed sense.

Another striking feature of the models is the invariant confinement of each chromosome arm to its own topological domain; the arms never intertwine or knot around one another (Fig. 3*c*). This is true despite the extensive, complicated interfaces between them, indicating that the chromosomes are not wound through the nucleoplasm at random.

Quantitative analysis of nuclear models

It was quickly realized that simple visual inspection, even of rotated stereo models, would be of limited usefulness and could in fact be misleading. Because these nuclei are far from identical, any further search for common features requires translating the models into a form which can be examined objectively. By analysing various parameters that describe properties of the modelled chromosomes, one can extract their salient structural features. The detailed derivation of these parameters and their plots is given elsewhere¹⁶. We describe here the important architectural features revealed by such an approach. These include definable chromosome folding patterns, specific chromosome contacts with the nuclear envelope, and, less firmly established, a hierarchy of preferred chromosome arrangements within the nucleus. In all cases, these elements can be referred to specific cytogenetic sequences, making the investigation of structural organization at once more precise and more readily related to functional aspects of the nucleus.

Folding patterns of individual chromosomes. As the chromosome cable folds up in three dimensions, certain points which are well separated along the axis of the chromosome may approach each other quite closely in space. Such interactions

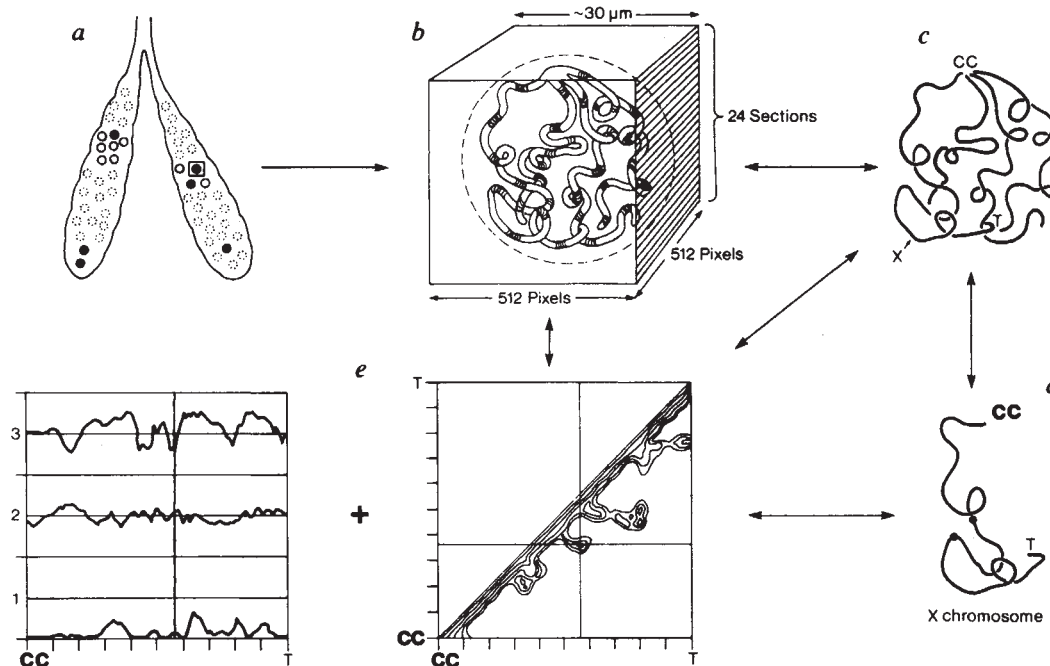


Fig. 2 The overall scheme of the study. The structural information of each nucleus was analysed *in situ*. *a*, The sample gland: ○, nuclei for which data were collected and preliminary models built; ●, nuclei used in this study. Fluorescently stained chromosomes were imaged using a high-resolution objective lens with a shallow depth of focus. Incrementing the focus in regular steps through a nucleus effectively sliced it into a stack of images (*b*). The pathways of the different chromosomes were then traced using the program IMP¹⁵, which moves a cursor within the data stack and uses it to place points along each arm. The vectors connecting these points defined a 'stick-figure' model (*c*). The model lines traced over the data set (see Fig. 1*b*) indicate to which path each piece of chromosome belongs, simplifying the cytological determination. Typically 25–35 bands, distributed all along the arm, were noted on the model. This was done for each arm. Chromosome pathways were then analysed for several properties, which are represented in a series of graphs (*e*). After the axes were normalized to the known banding pattern, these graphs were used to compare chromosomes from different nuclei. A cross-hair (shown in *e*) marks the positions in this plot which correspond to locations on the model. The regions in the model which gave rise to the targeted feature are marked in *d* by the two dots. CC, Chromocentre; T, telomere. The structural information of each nucleus goes through three successive abstractions: nucleus to image stack, then to curves delineating the chromosome paths, and finally to parameters describing properties of these curves. The computational analysis is focused on this final level, but any feature can be evaluated at any level.

can be visualized graphically in an intradistance map²². The distances between the three-dimensional positions of all pairs of points on a chromosome are measured; that distance is then subtracted from a cutoff distance and the resulting value is plotted in a two-dimensional map. Distances larger than our cutoff distance of 8 μm are plotted as zero in the map. In these plots, both axes represent cytological position along the chromosome, the centromere being at the origin. The value plotted in an intradistance map is greatest for points which are close together and linearly approaches zero for larger separations. An interaction between points which are not immediately contiguous along the arm will appear as a non-zero region located off the diagonal in the map (illustrated in Fig. 2*d,e*). Note that distance is measured from the chromosomal midlines defined by the model and that the chromosomes are, on average, 3–4 μm wide.

At first inspection, the intradistance maps of the same chromosome in different nuclei seem quite divergent. Nevertheless, common features in the set of six intradistance maps for a given chromosome arm are found when an appropriate statistical operation is performed on the collection. The operation we use calculates a generalization of the median value, called the rank order value; the rank = *n* value is the *n*th highest value in a distribution of values. The value for each point in a rank plot is derived from the distribution of values of the equivalent point in the original maps. In a rank = 4 plot prepared from a set of intradistance maps, each non-zero patch marks an area of conserved intrachromosomal apposition; at least four of the six nuclei studied have the corresponding chromosome regions apposed as close as or closer than is represented in the patch. Conserved regions are found throughout all of the arms. In Fig. 4, for example, a rank-ordered intradistance plot of the X

chromosomes shows a series of bars extending perpendicular to the diagonal. This kind of graphical feature is the hallmark of a loop in the model path and can be characterized by both its length and its location on the chromosome (Fig. 4*b*). A series of such loops is seen in the consensus structure of X. By evaluating such statistically ordered graphic signatures for each chromosome, it is possible to obtain an idea of their preferred configurations. The patterns are rather more complicated for the other chromosomes, and we are in the process of determining precisely what folded structures are represented in each. The results of this analysis will be published elsewhere. The data indicate that a basal set of characteristic local conformations is available to each arm which is nonetheless compatible with considerable freedom in the global structure.

Spatial relationships between different chromosomes. In contrast to the above intradistance plots, pairwise comparisons between different chromosome arms (interdistance plots) reveal very few, if any, specific interactions in this collection of nuclei. Only the 3L versus 3R diagram (at rank = 3) has an area of contact which is moderately conserved, centred at 72CE of 3L and 88AB of 3R (data not shown). The interdistance plots can still be used to obtain an idea of the nearest-neighbour relationships of the five chromosome arms for each nucleus. This is done simply by counting the number of close approaches in each pairwise comparison plot. Certain arrangements, although not unique, are seen much more frequently than others. The clearest examples are: the arms of a given metacentric chromosome are usually next to each other, 3L is always far away from 2R except at the telomeres, and most of the X is well separated from 3R.

These preferences are confirmed in a related set of plots. Here, we consider each point along a particular chromosome,

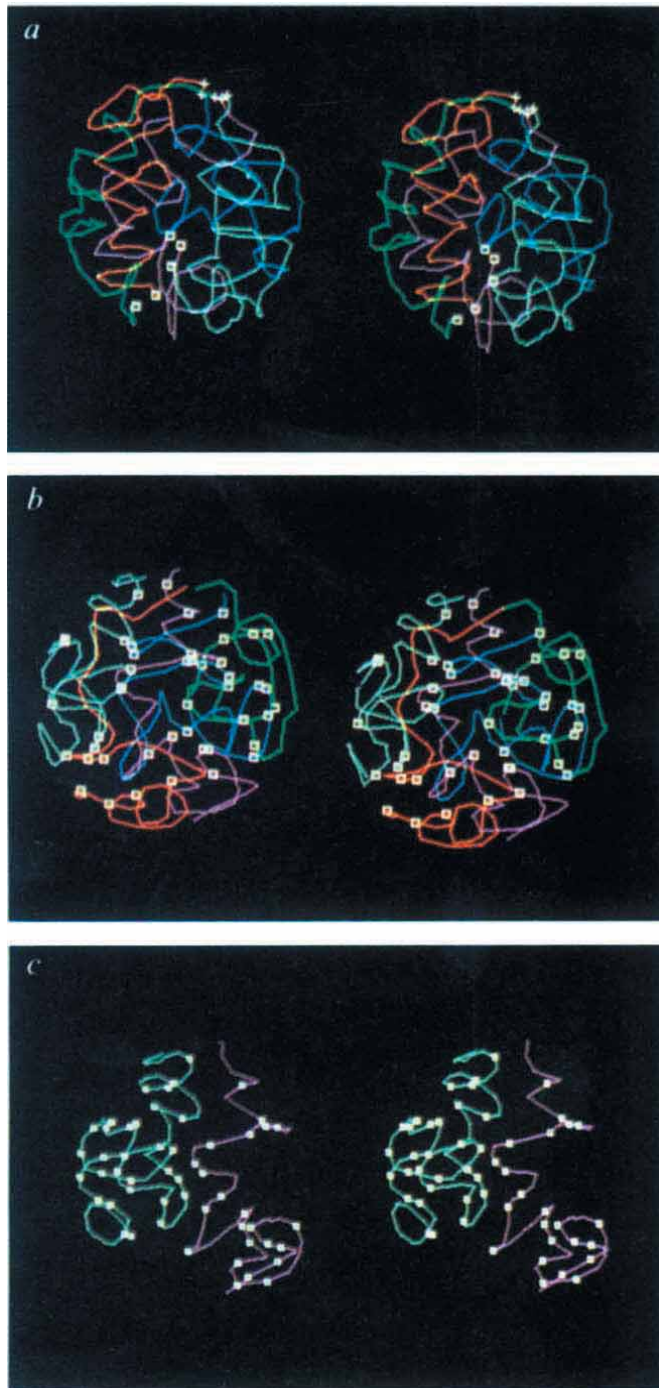


Fig. 3 *a, b*, Stereo pairs of two of the nuclear models. (Note that stereo glasses will reverse the handedness.) Corresponding chromosomes in the different models are assigned the same colour (green = X, orange = 2L, blue = 2R, purple = 3L, light blue = 3R). Models can be manipulated in several ways for visual inspection including rotation in near real time, selective blacking-out of sectors and chromosomes, and marking of cytological features. In *a* the telomeres (■) and centromere termini (+) are indicated to highlight the Rabl orientation³⁰. In *b* the active puff sites for glands at this developmental stage have been indicated by small squares⁴⁰. *c*, The 3L and 3R chromosomes from *b* are shown with all cytologically identified sites marked with small squares. Confinement of individual chromosome arms to separate topological domains is illustrated in this view; while the chromosomes press together quite closely they do not wrap around one another.



Fig. 4 This plot was used to evaluate the most common folding motifs which characterize the packaging of the X chromosome in the nuclei; it was derived as follows. First, the intradistance plot²² for each of the six X chromosomes was determined from its three-dimensional model. In such a plot the ordinate and abscissa are identical, representing cytological position along the chromosome, the centromere at the origin; what is displayed (*a*) is a cutoff distance (8 μ m) minus the distance in space between each pair of points on the chromosome, distances larger than the cutoff being plotted as zero. Contour lines were drawn immediately at every step of 1 μ m from the cutoff at zero up to the maximum at 8 μ m. Points immediately contiguous along the axis of the chromosome are necessarily close together in space; these together form the diagonal region in the plot which has the maximum value. Non-zero areas off the diagonal represent regions on the chromosome which are close in space and thus indicate the chromosome's folding pattern (see Fig. 2*d,e* for example). The second step was to consider the plots for all of the X chromosomes together in order to find their common features amidst the 'conformational noise'. The values at each position in the six original intradistance plots were sorted in order of descending value. The largest value was given a rank = 1, the smallest a rank = 6. A 'rank-ordered' plot was then composed using for each position in the two-dimensional plot the value that falls in the chosen rank. The plot shown uses the rank = 4 values for all positions in the graph, thus in four out of six nuclei, the chromosome points represented at each position are as close as or closer than shown in the plot. The bottom part of the figure (*b*) represents our interpretation of the rank-ordered plot for the X chromosome, showing the location of folding features. Feature centre (v), interior (---), ends (###). The features centred at the start of numbered divisions 3, 5, 9 and 12 are typical loops or foldbacks. Note that some, but not all, of them overlap.

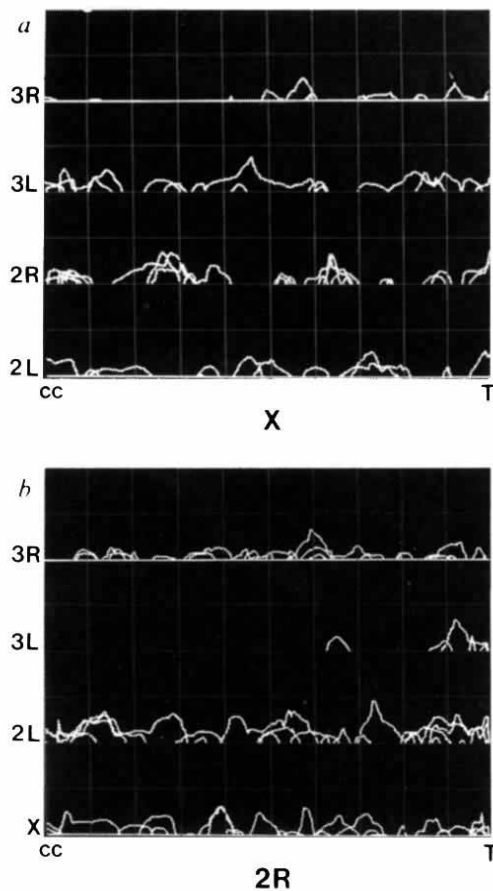


Fig. 5 Proximity of different chromosomes. *a*, Each plot shows the fraction of the indicated arm which falls within a $5\text{ }\mu\text{m}$ radius of each point on X. The vertical scale (two squares) equals 40% of the full length of an arm. Cytological position on the X chromosome is plotted along the abscissa: cc, chromocentre; T, telomere. Each graph includes plots from all six nuclei. *b*, The analogous set of plots for 2R. Evident in these plots is the preference of certain arms for particular neighbours, for example 2R versus 2L. In some cases there is a restriction of the apposition to subchromosomal regions of one or the other arm, as in X versus 2R in *a*.

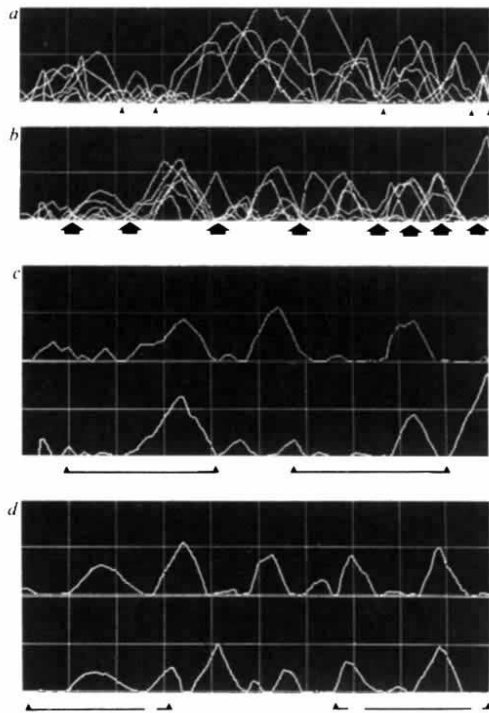


Fig. 6 *a-d*, Plots of the distance from cytological points along a chromosome to the nuclear surface. Full scale on the vertical axis is $10\text{ }\mu\text{m}$. Each horizontal division corresponds to two numbered cytological divisions, with the chromocentre at the left. Conserved contacts are indicated by arrows. *a*, Superimposed graphs for the six 2L arms. *b*, Superimposed graphs for the six X chromosomes. *c*, *d*, Several of the contributing X chromosome plots are shown individually. $\blacktriangle$ — $\blacktriangle$ Indicates regions with strong similarities. The surface is represented by a convex polyhedron tangential to the model at each of its facets, as described in the text. A simple algorithm for generating the facet planes is described by Mathog¹⁶. The distances plotted here were measured from each point along the chromosome arm to the closest point on the polyhedral surface.

and ask how many points from a different chromosome fall within a certain distance from it. For example, in Fig. 5*a*, points along the X chromosome are plotted along the abscissa. The value associated with each point represents the number of points on the interpolated path of another chromosome which fall within $5\text{ }\mu\text{m}$ of that position on the X; results for all six nuclei are superimposed. There is a distinct grouping of apposed sites in the plot of X versus 2R, suggesting specific and limited regions of X which interact with 2R. However, for the reverse comparison, that is, each point along 2R versus all points in X, no specific regions of interaction on 2R are noticeable (Fig. 5*b*, bottom curve). We interpret this to be a consequence of the particular folding of the X chromosome and the interface of this folded figure with the 2R domain; if the X generally exposes certain portions towards 2R, circumscribed areas of contact on X would be expected. 2R, on the other hand, may place a greater variety of sites towards its X neighbour.

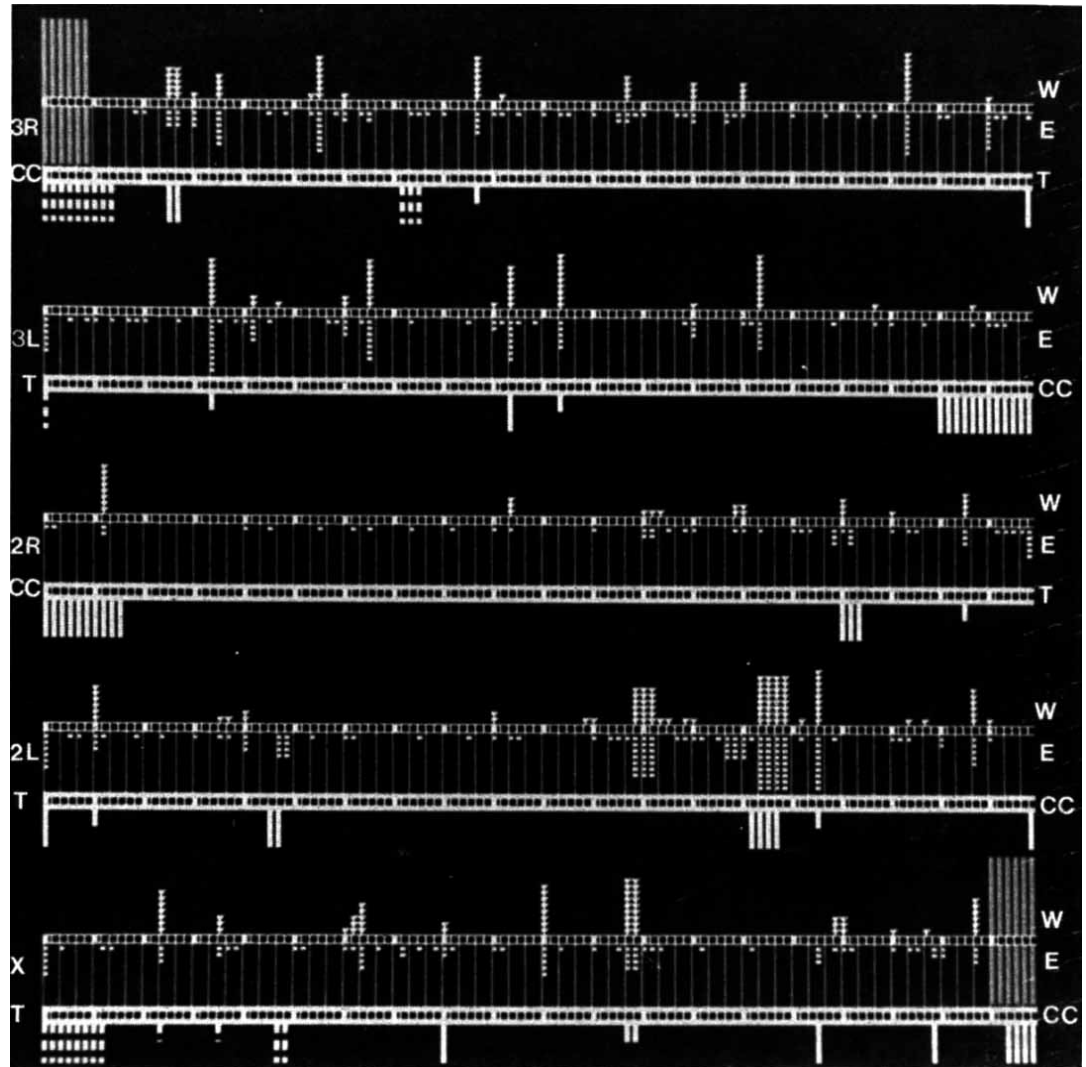
It appears that the chromosomes are packaged nonrandomly within the nucleus but without a determined set of pairwise interactions with specific sites on their nuclear neighbours. A caveat to this interpretation is that we have implicitly equated specific 'apposition' with 'interaction'. While this is probably valid in most instances, it does dismiss longer range and indirect interactions. For example, it is well known that ectopic fibres can sometimes reach enormous lengths, particularly in *Chironomus* where fibres as long as $45\text{ }\mu\text{m}$ have been observed in unsquashed nuclei²³.

Arrangement of chromosomes in relation to the nuclear surface.

The role of the nuclear envelope has been emphasized in several models of nuclear organization²⁴⁻²⁶. Evidence supporting these models includes surface associations of chromosome ends^{27,28}, ectopic fibres²³, and replication complexes²⁹. Accordingly, we were interested in determining how the chromosomes in our models were positioned with respect to this structure.

The chromosomes are intimately associated with the nuclear envelope at many points over its entire inner surface; this can be verified directly using phase or Nomarski optics or by specifically staining the envelope. The parts of the chromosomes closest to the envelope thus define a convex boundary equivalent to the inner surface itself. This boundary is evident in Fig. 1. A close approximation to this surface can be constructed from several hundred planes which are tangential to the model¹⁶. This is equivalent to inscribing the model within a multifaceted polyhedron. The minimum distance from this surface to each point along a chromosome is then calculated and plotted (Fig. 6). Figure 6*a* and 6*b*, for example, shows the plots of the six corresponding 2L and X chromosome arms, respectively. There are many regions that vary quite freely. On the other hand, points that show a close convergence of lines indicate a nonrandom relationship of the loci to the nuclear surface. These relatively fixed points are almost all located on the nuclear surface and are nearly all at loci have previously been singled out for several structural peculiarities. These special features, collectively referred to as intercalary heterochromatin, include

Fig. 7 Features characteristic of intercalary heterochromatin compared with conserved surface contact sites. Each horizontal scale is divided into the 120 lettered cytological subdivisions for each chromosome arm; the first square of each numbered division is filled. Weak points (W) and ectopic pairing points (E) are shown on top scale; these data are from ref. 12. Their frequencies of observation have been binned here into steps of 10% (W) and 1% (E) (intermediate values rounded to next highest bin). Grey bars indicate regions where they made no observation. Our surface contact sites are shown on the corresponding bottom scales. Large solid bars indicate surface apposition in six out of six nuclei, small solid bars in five out of six nuclei with the sixth at a peak in the distance to surface plot, and large broken bars mark all other cases of five out of six. The pair of small broken bars at 3C and 4D indicates that either site is on the surface to the exclusion of the other. The chromocentre (CC) and telomeres (T) are marked. The resolution of the surface sites is about one lettered subdivision. Data for the 2L and X chromosomes are shown in Fig. 6a, b.



frequent ectopic pairing, late replication, a strong tendency to split or break in squash preparations, and a proclivity for chromosome rearrangement^{11,12}. Figure 7 summarizes these contact sites and compares them with regions of the chromosomes containing intercalary heterochromatin. There is a strong correlation between the conserved surface sites and the intercalary heterochromatin regions. The surface contacts may result from the formation of particular chromosome configurations, or they may actively determine these configurations; we cannot distinguish between these alternatives on the basis of our data.

When one compares the plots of one particular arm from different nuclei, some regions (as much as two-thirds of the total length) often show strikingly similar relationships to the envelope; Fig. 6c and d give two examples of this. The simplest explanation for such strong pathway homologies between segments of chromosome arms in different nuclei is that there is a small set of folding conformations which can be assumed by particular segments; thus in any group of nuclei several different but nonrandom patterns will be observed. In any case the plots do show that many parts of the chromosomes do not haphazardly contact the membrane.

Discussion

From the data presented here, a detailed three-dimensional picture of an interphase nucleus, with features down to the level of the specific cytogenetic banding pattern, begins to emerge. Overall, the architectural arrangement retains a significant

degree of flexibility, but at the local level the configurations and dispositions of the chromosomes are quite narrowly circumscribed. Chromosomes fold up in highly characteristic ways, usually in a series of specific loops of definable length and position which often form roughly helical structures. Their centromeres, fused into the chromocentre, stick to the nuclear envelope's inner surface probably within a restricted area while their telomeres tend to congregate around the opposite nuclear pole (the Rabl orientation³⁰). The curling and twisting arms respect strict topological boundaries with respect to one another, in no instance wrapping around a neighbour. Some preference for certain nearest-neighbour arrangements is also seen. Finally, several nonrandom contacts with the inner nuclear surface are made by chromosomes as they pack into the outer regions of the nucleus.

The confinement of chromosome arms to separate topological domains is a striking result and one which does not necessarily follow from the other features of order which we have found. How might this strict separation be maintained? The way in which the individual arms fold up, perhaps involving ectopic fibres, may preclude their interweaving. Another possibility is the specific scaffolding of individual arms within the nuclear sap to a protein matrix or to the nuclear envelope.

Perhaps the domain restrictions can be best understood as a stable arrangement of the chromosomes established shortly after the completion of the last embryonic mitosis. The Rabl orientation is generally thought to reflect the configuration of the

chromosomes in the preceding telophase³¹. The topological independence of the chromosome arms can be viewed as a natural extension of this idea. There are several precedents for the limitation on interphase chromosome movement that would be required by such an interpretation³²⁻³⁴.

The specific chromosome-envelope touchpoints almost all map to sites of intercalary heterochromatin¹² (Fig. 7). Heterochromatin is known to aggregate near the nuclear surface in certain tissues^{35,36}. Skaer *et al.*³⁷ noted that the patches of heterochromatin are tightly attached to the nuclear membrane. It is possible that the sites catalogued here may also serve as specific anchor points to the nuclear envelope. Approximately 50% of the sites of strong ectopic pairing (≥ 3 bars in Fig. 7), neglecting the extreme proximal regions of each arm, are found to be conserved surface points. Why one subset of intercalary heterochromatin sites should be preferentially on the surface while the rest are not is unknown. Because of the limited number of nuclei included in this study we cannot distinguish by our criteria between sites which are not strongly conserved and sites which are not conserved at all.

From the intradistance plots, conserved regions of folding can be observed within each of the individual arms, the most common motif being the loop. The loops could be the consequence of ectopic fibre connections or simply the mechanical characteristics of the synapsed chromosomes themselves. The pattern of attachment points on the envelope may also be important. A preliminary survey of weak points in the original data sets strongly argues for some kind of mechanical role for these sites as they are often at very tight bends or strong twists.

The picture outlined here is probably a rather conservative estimate for the order which actually exists *in vivo*. A fundamental assumption of this study has been that if a specific arrangement of chromosomes exists, it should be reiterated in most or all the nuclei of a given tissue. There are, however, almost certainly some differences between cells in different portions of the gland³⁸. Furthermore, the gland at this developmental stage (about PS7-8) is going through a conspicuous series of changes in its puffing patterns and also in its gross morphology^{39,40}, and unless these changes are entirely synchronous, additional cell-cell heterogeneity will be introduced.

Other possible sources of variation must be considered. If the chromosome conformations are very labile, we may have caught the different nuclei of the sample gland at various points in a spectrum of conformations. To test this, a living gland (by the criterion of Trypan blue exclusion at the end of the test period) was continuously viewed by phase contrast optics over several hours. Pictures were taken at regular time intervals. No change in position of any chromosome region could be detected when the frames were compared (data not shown). This suggests that large rearrangements do not take place in the living gland over a period of several hours. Neither could any disruption of the nuclear contents be detected over a long period of time in the buffer conditions used to collect the image data. Moreover, these glands have not been fixed and thus are not subject to the well documented artefacts which fixation regimens can create⁴¹.

The conclusions one draws from such models do not depend on who constructs them even though different model builders' placement of points within the 3-4 μm -thick chromosomes may vary slightly. One nucleus was modelled independently by two of us. The resulting reconstructions differed in only trivial ways from one another, and more importantly, the resulting quantitative plots were virtually indistinguishable.

A more difficult problem in this analysis concerns the small sample size. There is no good foundation for predicting a random set of conformations as a statistical control, as we know very little about the boundary conditions. The restricted volume of the nucleus, exacerbated by the excluded region occupied by the nucleolus; the centromeric fusion of all the arms; limits on the mechanical deformation of the chromosome cable; and other constraints all restrict the ways in which the chromosomes can fold. Defining these boundary conditions is, of course, a major

goal of our analysis. We are analysing more data both for this purpose and for reducing the problem of limited sample size.

There are several means by which a nonrandom chromosome organization within the nucleus can be rationalized^{24,29}. As discussed above, at least certain aspects can probably be best understood as relics of the last embryonic mitosis of these nuclei. Also, certain folding patterns and chromosome arrangements may simply fit more easily into the confined space of the nucleus. An additional coercive force on interphase chromosome arrangement could be the requirement that chromosomes in germ-line tissues be able to pair with their homologues during meiosis^{24,42-44}. Maintaining a defined spatial arrangement of chromosomes would greatly facilitate the orderly pairing and segregation of homologues, and somatic tissues could conceivably bear vestiges of this order. Certainly the somatic nuclei of the dipterans, with their tightly paired homologues, attest to this possibility. Nonrandom arrangements of homologues and non-homologues is now supported by a wealth of cytological data in a variety of species^{9,43,45}, although the evidence in mammals remains controversial^{33,46}.

A more radical rationale for determined configurations of chromosomes in the nucleus is that they are involved in gene expression. As few tools have previously been available to test this idea, the evidence is rather scant. We can now identify a large number of loci in their unperturbed three-dimensional context, providing a means of studying this problem. One straightforward approach would be to analyse the locations of known active loci as a function of developmental time. The glue protein genes and the ecdysone-induced puffs would be the first choices in such experiments. Indeed we have begun to measure the spatial relationships of the latter group in the present data set. Several other issues are also being examined, among them the spatial distribution of specific chromosomal proteins (as revealed by monoclonal antibodies) associated with active sites^{47,48}.

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Messenger RNA from human brain induces drug- and voltage-operated channels in *Xenopus* oocytes

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*Sodium channels and receptors to serotonin and kainate were 'transplanted' from human brain into frog oocytes, by isolating messenger RNA from a fetal brain, and injecting it into *Xenopus laevis* oocytes. The mRNA was translated by the oocyte and induced the appearance of functional receptors and channels in its membrane. This approach renders drug- and voltage-operated channels of the human brain more amenable to detailed study.*

FOR obvious reasons, it is important to know how neurotransmitter receptors and channels function in human brain neurones; but these cells are not easily amenable to the necessary experimental approaches. One possibility would be to culture human brain cells^{1,2} and study them with electrophysiological and biochemical techniques^{3–6}. Alternatively, one might purify the receptor and channel proteins from human neuronal membranes, and study their function after incorporation into artificial membranes, as has been done with other systems^{7,8}. We decided on a different approach, one that may allow us to study not only the functioning of receptors and channels, but also the way in which their constituent proteins are synthesized, processed and incorporated into the cell membrane. Essentially, the method consists of isolating messenger RNA (mRNA) from the human brain, and injecting it into oocytes of *Xenopus laevis*. This induces the appearance of receptors and channels in the oocyte membrane, in the same way as was done previously with mRNA from chick⁹ or rat^{10–12} brains.

Membrane channels induced by mRNA

Injection of mRNA derived from fetal human cerebral cortex into *Xenopus* oocytes caused the synthesis, and incorporation into the membrane, of channels which could be activated either by drugs or by voltage changes. These included voltage operated sodium channels (Figs 1, 2), and receptor-channel complexes activated by serotonin (Fig. 3) and kainate (Fig. 4). All of them resulted from the injection of exogenous mRNA, since most injected oocytes gave responses, whilst control (non-injected) oocytes from the same donors did not show responses (Table 1).

Two further lines of evidence indicate that the proteins forming these membrane channels arose directly from the translation of the human mRNA by the oocyte, and not because the injection of foreign mRNA caused the oocyte to transcribe the appropriate messengers from its own genome. First, oocytes developed the voltage- and drug-activated responses even after they had been exposed continuously to actinomycin D (50 $\mu\text{g ml}^{-1}$) to inhibit synthesis of mRNA¹³. Second, the responses still developed in oocytes which had been enucleated^{12,14} before injection of human mRNA.

Voltage-activated sodium current

A few days after injecting the oocytes with human cerebral cortex mRNA, depolarizing pulses elicited an inward membrane current (Fig. 1a). In contrast, non-injected oocytes from these donors showed predominantly passive (ohmic) currents. Figure 1 illustrates the current-voltage relationship for the inward current. The current first became detectable when the membrane potential was stepped from -100 to about -40 mV, and increased with increasing depolarization to reach a maximum at about -10 mV. With further depolarization the current declined, and was reduced to about zero at a potential of $+60$ mV.

The peak amplitude of the inward current elicited by depolarization was reduced if external sodium was partially substituted with Tris or tetraethylammonium ions. Furthermore, tetrodotoxin (TTX) readily abolished the inward current (complete block with 300 nM, half block with 10 nM). All this indicates that sodium is the main ion responsible for the inward current. Manganese (5 mM) also reduced the inward current, but this effect probably arises from a change in the characteristics of the sodium channels, rather than from a blockage of calcium channels^{11,15}.

The decay of the sodium current in oocytes injected with human cerebral cortex mRNA often showed two time courses. For example, in the upper record in Fig. 2a, the current shows an initial rapid decline, followed by a slower decay. During repetitive activation, the slow component of the decay 'fatigued' more readily than the fast component. This is evident in Fig. 2a, where two depolarizing pulses of 900 ms duration to a potential of -10 mV were separated by an interval of 100 ms. The slow component was strongly reduced during the second pulse, whilst the fast component appeared little changed.

Sodium currents induced by rat brain mRNA show a similar, two-component decay¹¹, but with a time course approximately three times faster. Mean values for the half decay time of sodium currents measured in oocytes from the same donor (at -10 mV and 12 – 15°C) were: human cortex, 13.7 ± 1.3 ms (s.e.m., 18 oocytes); rat brain, 4.8 ± 1 ms (6 oocytes).

Table 1 Drug- and voltage-activated membrane currents induced in *Xenopus* oocytes following injection of mRNA

	Human cerebral cortex	Human cerebellum	Rat brain	Control
Sodium current	68 ± 12 (30)	6 ± 1.7 (15)	332 ± 40 (23)	0 (8)
Potassium current	0 (23)	0 (14)	331 ± 57 (23)	0 (8)
T_{out} current	22 ± 3.6 (29)	17 ± 5 (14)	341 ± 107 (24)	15 ± 3.5 (8)
Serotonin	60 ± 12 (19)	2 (8)	625 ± 126 (4)	0 (6)
	[10 ⁻⁵ M]	[10 ⁻⁵ M]	[10 ⁻⁷ M]	[10 ⁻³ M]
Kainate	12 ± 4 (20)	3 ± 1 (11)	18 (4)	0 (6)
	[10 ⁻³ M]	[10 ⁻³ M]	[10 ⁻⁵ M]	[10 ⁻³ M]
GABA	0 (12)	0 (7)	250 (1)	0 (6)
	[10 ⁻³ M]	[10 ⁻² M]	[10 ⁻³ M]	[10 ⁻³ M]
			277 ± 143 (4)	0 (6)
			[10 ⁻⁴ M]	[10 ⁻³ M]

Numbers give peak current responses in nA, with standard error of mean, and number of oocytes in parentheses. In the case of drug-activated currents, measurements were made at a clamp potential of -60 mV, and the drug concentrations are given in square brackets. The voltage-activated sodium current was measured as the peak current elicited by depolarization to a potential of -10 mV from a holding potential of -100 mV. Similarly, the potassium current and the transient outward (T_{out}) current were measured for depolarizations to, respectively, +40 and 0 mV. All oocytes, including controls (non-injected), were obtained from the same two donors. Temperature, 12–15°C.

Effects of toxins

The sodium channels induced by human mRNA in *Xenopus* oocytes were blocked by TTX and were affected by veratrine, as are the sodium channels in nerve membranes¹⁶. For instance, the sodium current is normally inactivated almost completely during long depolarizing pulses (Fig. 1a). In contrast, after veratrine some inward current remains during pulses lasting many seconds. Figure 2b, c shows currents elicited by depolarizations to 0 mV before and after adding veratrine (0.5 mg ml⁻¹) to the perfusion fluid. In the presence of veratrine a maintained inward current persisted throughout the depolarization, while the initial transient inward current was slightly smaller and showed a faster decline (Fig. 2b). The most striking effect was, however, seen when the oocyte was repolarized. Normally, no sodium-dependent tail currents were apparent when the membrane was repolarized, but a few minutes after adding veratrine large and slowly decaying current relaxations were recorded (Fig. 2b,c). Both the inward tail current and the maintained inward current during depolarization were blocked by TTX. Aconitine is also known to modify sodium channels in nerve membranes¹⁷ but caused no obvious changes in the sodium currents in the oocyte, even at a concentration of 5 × 10⁻⁴ M.

Scorpion venom (*Leiurus quinquestriatus*) reduced the maximum peak sodium current evoked by depolarizing pulses and also caused the appearance of a TTX-sensitive current which was maintained throughout the depolarizing pulse. In contrast to veratrine the maintained current caused by scorpion venom was rapidly turned off when the membrane was repolarized. The effects of scorpion venom resembled those seen in frog and squid axons^{18,19}.

Although large (~1 μA) sodium currents could be recorded from oocytes injected with human cortex mRNA it was not normally possible to elicit action potentials by injecting depolarizing current pulses. This was mainly because inactivation of the sodium current was rapid compared with the electrical time constant of the oocyte. However, when inactivation was reduced by veratrine, it became possible to elicit regenerative responses which were blocked by TTX (Fig. 2d).

Serotonin receptor channels

Application of serotonin to oocytes injected with human cortex mRNA elicited membrane currents, whilst control oocytes from the same donors showed no responses, even with high drug concentrations (Table 1). Striking features of the serotonin responses were the long latency to onset, and the slow oscillatory

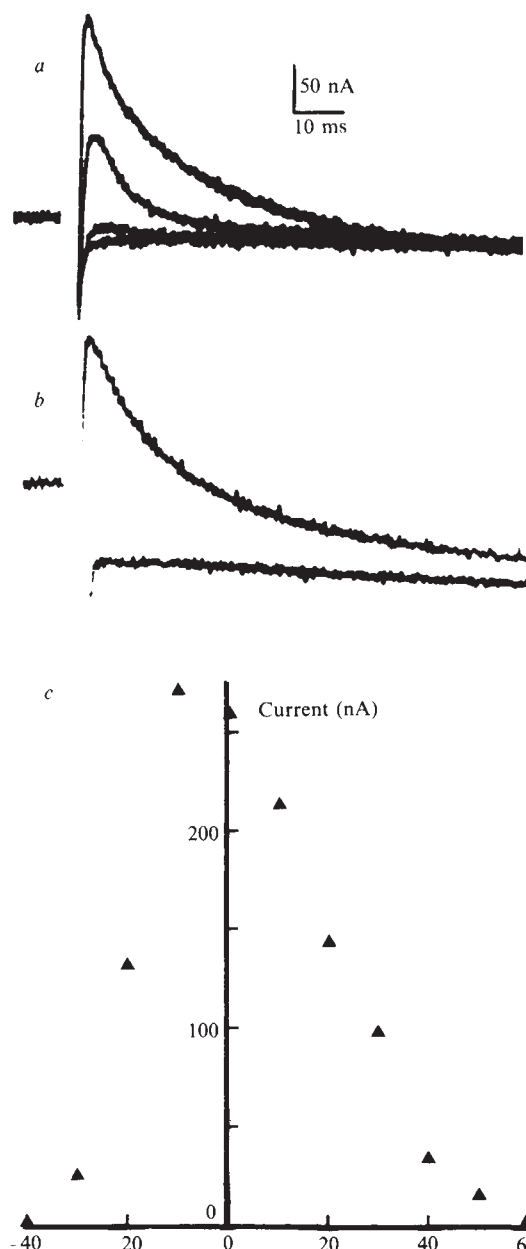


Fig. 1 Voltage dependence of the inward sodium current, in an oocyte injected with human cerebral cortex mRNA. The holding potential was -100 mV in all cases. Temperature, 15.5°C. *a*, Membrane currents elicited by depolarization to potentials of -50, -30, -20 and -10 mV. The response increased as the potential was made more positive. *b*, Currents elicited from the same oocyte at potentials of +10 and +40 mV. The inward sodium current was smaller at +10 mV than at -10 mV, and was greatly reduced at +40 mV. *c*, Current-voltage relationship for the peak amplitude of the sodium current, measured in the same oocyte. Measurements were made by subtracting the currents elicited at each voltage before and after addition of TTX (313 nM) to the perfusion solution.

Methods: Procedures for isolation of poly(A)⁺ mRNA and its injection into oocytes of *X. laevis*, were as described previously^{10-12,30}. Rat brain mRNA was obtained from Wistar rats, and human brain mRNA was isolated from the cerebral cortex of a 15-week-old fetus. Oocytes were voltage clamped and perfused with Ringer solution at 11–15°C (refs 9–12). Most experiments were made on oocytes which had been treated with collagenase to remove follicular and other enveloping cells²¹.

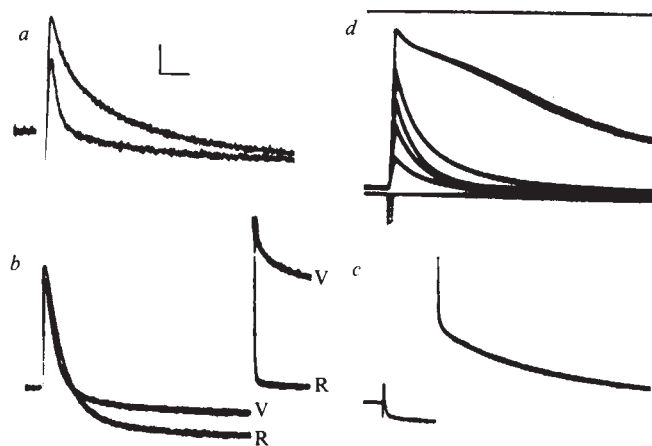


Fig. 2 Effects of repeated stimulation and of veratrine on the decay of the sodium current in oocytes injected with human cerebral cortex mRNA. *a*, Superimposed records showing membrane currents elicited by a pair of depolarizing pulses to -10 mV, from a holding potential of -100 mV. Pulse duration was 900 ms and the pulses were separated by an interval of 100 ms. The oocyte had been injected with human cortex mRNA and was treated with collagenase. Temperature, 15°C . Calibration bars, 50 nA and 10 ms. *b, c*, Effects of veratrine on the sodium current elicited by a depolarizing pulse to 0 mV. Calibration bars are 50 nA and 50 ms in *b* and 100 nA and 200 ms in *c*. Traces marked R and V in *b* were obtained respectively before and after adding veratrine (0.5 mg ml^{-1}), whilst the trace in *c* shows the tail current in the presence of veratrine on a slower sweep speed. *d*, Action potential elicited in the presence of veratrine from the same oocyte as in *b, c*. Traces show (from top to bottom) 0 mV potential reference; membrane potential; and current injected into the oocyte. A steady current was passed to hold the membrane potential at about -100 mV, and responses are shown to a series of depolarizing current pulses of increasing intensity. The largest pulse triggered a graded regenerative response. Calibration bars, 20 mV (membrane potential), 500 nA (current monitor) and 500 ms. Temperature in *b-d* was 12°C .

nature of the currents (Fig. 3). In these respects, the responses are quite different from the smooth currents elicited by activation of γ -aminobutyric acid (GABA), kainate or nicotinic acetylcholine (ACh) receptors incorporated into oocytes following injections of the appropriate mRNA^{9,13,20}. They do, however, resemble the oscillatory currents elicited by serotonin in oocytes injected with rat brain mRNA¹⁰, as well as the currents elicited by activation of muscarinic ACh receptors²¹ and glutamate receptors¹². In the present experiments, application of ACh and glutamate to oocytes, which showed good sensitivity to serotonin following injection of human mRNA, did not elicit any appreciable currents. Thus, the serotonin responses probably involve a specific serotonin receptor.

The serotonin response was abolished by low concentrations ($<10^{-6}\text{ M}$) of methysergide, a serotonin receptor antagonist²² (Fig. 3*a, b*). The blocking action of methysergide appeared to be either irreversible, or only slowly reversible, since responses to serotonin were still blocked even after washing for an hour or more.

In contrast to the powerful blocking action of methysergide, the serotonin antagonists cyproheptadine and ketanserin²³ did not appreciably reduce the size of the response to 10^{-5} M serotonin when applied at a concentration of 10^{-6} M . Lysergic acid diethylamide ($2 \times 10^{-6}\text{ M}$) reduced, but did not completely abolish, the response to $5 \times 10^{-7}\text{ M}$ serotonin (Fig. 3*c, d*).

Membrane potential and serotonin current

The oscillatory currents induced by serotonin in oocytes injected with human cortex mRNA decreased in size as the membrane was depolarized and inverted direction at a potential of about

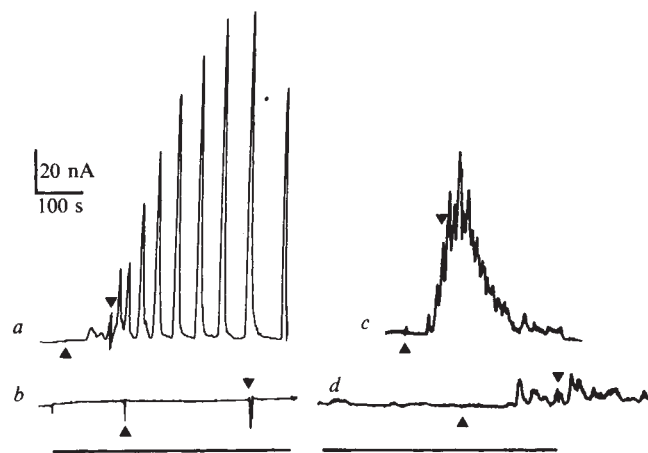


Fig. 3 Oscillatory membrane currents elicited by serotonin in oocytes injected with mRNA from human cerebral cortex. All records were obtained with the oocytes clamped at a potential of -60 mV so as to be away from the equilibrium potentials for sodium, potassium and chloride ions²¹. Serotonin was applied by bath perfusion, beginning and ending as indicated by the arrows. *a*, Response to serotonin recorded from an oocyte in normal Ringer's solution. *b*, Lack of response when serotonin was applied in the presence of methysergide. The bar indicates the duration of methysergide application. The concentration of serotonin was 10^{-6} M in both records, and the concentration of methysergide was 10^{-5} M . Temperature 14°C . *c, d*, Reduction of size of the serotonin response by lysergic acid diethylamide. Serotonin was applied at a concentration of $5 \times 10^{-7}\text{ M}$ in both records, and lysergic acid diethylamide was added at a concentration of $2 \times 10^{-6}\text{ M}$ for the time indicated by the bar in *d*. Temperature, 11°C .

-20 mV, which corresponds to the chloride equilibrium potential in *Xenopus* oocytes²¹. The equilibrium potential of the serotonin currents in oocytes injected with rat brain mRNA is also similar¹⁰. Thus, chloride is the main ion flowing through the channels activated by serotonin.

Depolarization beyond the equilibrium potential gave larger serotonin currents than corresponding steps in a hyperpolarizing direction, similar to the rectification seen with serotonin currents induced by rat brain mRNA¹⁰. This effect probably arose as a result of a voltage-dependent closing of the serotonin-activated channels, since a hyperpolarizing step applied near the peak of a serotonin-activated oscillation initially gave an increase in membrane current, which subsequently declined over a time course of a few hundred milliseconds.

Kainate receptor channels

Application of kainate to oocytes injected with human cerebral cortex mRNA elicited inward membrane currents (Fig. 4). These responses did not show oscillations, and were well maintained during kainate applications lasting several minutes. We did not observe clear responses to glutamate, even when applied at high concentrations (10^{-2} M) to oocytes which had a high sensitivity to kainate. This shows that in humans, as in rats¹², there is a kainate receptor which is distinct from glutamate receptors.

Currents elicited by different doses of bath-applied kainate are illustrated in Fig. 4. The minimal concentration required to give detectable responses was higher than for serotonin. For example, the oocyte illustrated in Fig. 4 gave a just detectable response at 10^{-5} M kainate, while a concentration of $5 \times 10^{-4}\text{ M}$ gave a maximal response. At low doses of kainate, a doubling in the concentration more than doubled the peak size of the response.

The reversal potential of the kainate-induced current (estimated by stepping the membrane to different potentials before and during steady kainate application) was about -10 mV. At voltages more negative than the equilibrium potential, the cur-

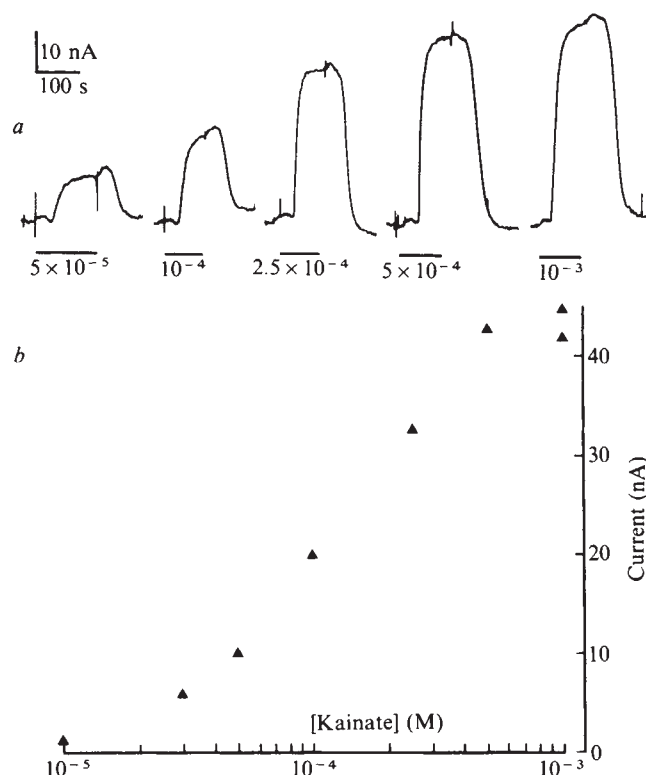


Fig. 4 Membrane currents elicited by kainate in an oocyte previously injected with human cortex mRNA. All records were obtained at a clamp potential of -60 mV. Temperature, 14°C . *a*, Responses to different concentrations of kainate, applied for the durations indicated by the bars. *b*, Dose/response curve for kainate, measured from the same oocyte as *a*. The peak current amplitude is plotted against kainate concentration on a semi-logarithmic scale.

rent-voltage relationship of the kainate-induced current was close to linear, which is similar to that of the kainate receptor induced by rat brain mRNA¹².

Human and rat membrane channels

Oocytes from two donors were injected with three different mRNA preparations, derived from fetal human cerebral and cerebellar cortices, and from rat brain. The extent to which these particular messenger preparations led to the expression of functional drug- and voltage-activated membrane channels differed markedly (Table 1).

Control (non-injected) oocytes showed no appreciable drug- or voltage-activated responses, except for a small transient outward current²⁴. In contrast, oocytes injected with rat brain mRNA gave large responses to serotonin, kainate and GABA and showed large voltage-activated sodium, potassium and transient outward currents (see also refs 10–12). The mRNA derived from human cerebral cortex was less effective. Responses to serotonin and kainate were present, as was the voltage-activated sodium current, but all were smaller than that found with rat brain mRNA. Furthermore, there were no detectable voltage-activated potassium and transient outward currents, nor were there any responses to GABA (Table 1) or to several other putative transmitter substances tested. Oocytes injected with mRNA from fetal human cerebellum showed little development of drug- or voltage-activated membrane currents.

Conclusions

Our results show that mRNA coding for proteins that make up voltage- and drug-operated channels in the human brain, can be effectively translated in *Xenopus* oocytes. Clearly, the cells in the brain contain many types of receptors and channels and we have so far managed to 'transplant' into the oocyte membrane only a few. Perhaps others were present and were not discovered

and we hope that other human mRNA preparations will yield still other receptors. Nevertheless, the results obtained so far with mRNA from chick⁹ and rat brains^{10,12} indicate that a given mRNA preparation produces a comparatively small number of functional receptors in the oocyte. It may be that these are preferentially expressed because the corresponding messages are more numerous, or because they have special features which render them more likely to be translated.

It is interesting that 'human' sodium currents were slower than 'rat' sodium currents. Pending confirmation with other mRNA preparations, this already suggests that sodium channels in the fetal human brain differ from those in the adult rat brain¹¹, or the embryo chick brain (K. Sumikawa, R.M. and I.P., unpublished). Moreover, since these channels were compared in oocytes from the same donor, it appears that the different kinetics of the sodium currents reflect differences in the proteins which make up the channels. Another interesting observation is that rat and chick brain mRNA induced transiently activated potassium channels in the oocyte membrane, while the mRNA from human brain did not. Furthermore, the relative sizes of the sodium and potassium currents in oocytes injected with rat brain mRNA varied greatly. All this suggests that sodium and potassium channels are encoded by different messengers which are not concomitantly expressed in the oocyte.

The effect of TTX, veratrine and scorpion venom show that, like other sodium channels^{16,25–29}, the human sodium channels have binding sites for these substances. The ineffectiveness of aconitine indicates that this substance may act at a different site from the other toxins examined. It could be that human brain channels do not have a site for aconitine, but it is also possible that this substance acts within the cell¹⁷ and that the oocyte membrane is poorly permeable to aconitine.

Perhaps the most important outcome of the present experiments was that we were able to induce functional human brain receptors to serotonin and kainate, and we hope that other receptors will soon follow. This opens the way to detailed studies on the mode of action of drugs on human receptors, and on the factors which control the synthesis and incorporation of receptors into the membrane. Ultimately, this knowledge may contribute to the alleviation of some forms of human brain dysfunction.

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Joining of V_{κ} to J_{κ} gene segments in a retroviral vector introduced into lymphoid cells

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V_{κ} to J_{κ} recombination occurred within a DNA construct introduced into cells in the form of a defective retrovirus. Analysis of one recombinant demonstrated that V_{κ} - J_{κ} joining can occur via inversion with a net loss of a few base pairs.

IMMUNOGLOBULIN genes are somatically assembled from widely separated DNA segments (see ref. 1 for a review). For κ light chains, recombination brings together two coding units to form the complete variable region portion of the gene: one of perhaps several hundred inherited V_{κ} gene segments joins to one of five J_{κ} segments in a reaction that occurs during B-cell development. The signals that allow recombination enzymes in the cell to recognize immunoglobulin gene segments and to fuse them are not defined, but in all cases rearranged DNA is found previously linked to a heptamer-spacer-nonamer of characteristic structure, suggesting a critical role for this DNA element in the joining reaction. A system which allows introduction into cells of defined DNA constructions that will then undergo the joining process would greatly facilitate the study of the sequence requirements and, more generally, the organizational and mechanistic features of this site-specific DNA recombination process. We report here the development of such a system.

In outline, we approached the problem by infecting a cell line that undergoes immunoglobulin gene rearrangement with a genetically engineered retrovirus containing a germ-line V_{κ} segment (linked to its heptamer/nonamer joining signal) and the five germ-line J_{κ} segments. We used a selectable marker to recover cells that had putatively rearranged the introduced substrate; we then cloned and sequenced one resident provirus to prove that V_{κ} - J_{κ} joining had occurred.

Substrate design

The selection procedure was suggested by a model we presented previously. Studies of κ gene rearrangement have shown that in addition to the $V_{\kappa}J_{\kappa}$ coding joint that is formed, it is often possible to detect additional DNA fragments with hybridization properties and sequence characteristics suggesting that they originate as reciprocal recombination products of V_{κ} - J_{κ} joining²⁻⁶. To explain the presence of such reciprocal joints, we proposed that some (and possibly all) V_{κ} elements are organized in germ-line DNA such that their transcriptional orientation is opposite to that of the J_{κ} elements and that inversions joining V_{κ} to J_{κ} would concomitantly generate the reciprocal joint⁴. On the basis of this idea, in our retrovirus construction we placed V_{κ} and J_{κ} in transcriptionally opposite orientations with a selectable marker between them. The marker was positioned in noncoding orientation relative to the promoter in the 5' long terminal repeat (LTR) and therefore was not expected to be expressed unless activated for expression by an inversional V_{κ} - J_{κ} joining event. The selection was designed to depend on an inversional rather than a deletional recombination for three reasons: to minimize background from rearrangements unrelated to V_{κ} - J_{κ} joining, to allow recovery of the coding joint as well as the noncoding reciprocal joint, and to test directly whether it is possible for V_{κ} - J_{κ} joining to occur via inversion.

A representation of the relevant region of the pMSVgpt-based plasmid⁷ constructed for these experiments is shown in Fig. 1 (top). The sequences designed to serve as the joining substrate were flanked by retroviral LTRs derived originally from

Moloney sarcoma virus (MSV). Between the LTRs were located (in the 5' to 3' orientation of an RNA transcript): the 5' splice site of MSV which is used by the wild-type virus to allow envelope gene expression; the packaging sequence of MSV⁸; the J_{κ} segments (in 3' to 5' transcriptional orientation); the *Escherichia coli* xanthine-guanine phosphoribosyl transferase (*gpt*) gene (in 3' to 5' orientation) and an inverted envelope gene 3' splice site. This was followed by a 5' to 3' oriented genomic $V_{\kappa}21-C$ segment derived from BALB/c DNA (provided by Dr S. Tonegawa). This particular V_{κ} segment has been identified as having rearranged into the active allele in the MOPC 321 myeloma^{9,10}. The DNA following the $V_{\kappa}21-C$ segment in the construct is irrelevant except for the 3' LTR and its associated primer binding site.

Retrovirus infection

The plan of the experiment is detailed in Fig. 2. The DNA construct was transfected into ψ -2, a fibroblast cell line that constitutively produces all of the murine leukaemia virus (MuLV) proteins but is unable to package its resident helper virus genome⁸. A co-transfected plasmid carrying a neomycin resistance gene allowed the isolation of stably transfected ψ -2 cells. The virions produced by the transfected cells were used to infect PD cells, a lymphoid cell line that undergoes continuous rearrangement of its κ gene segments⁴.

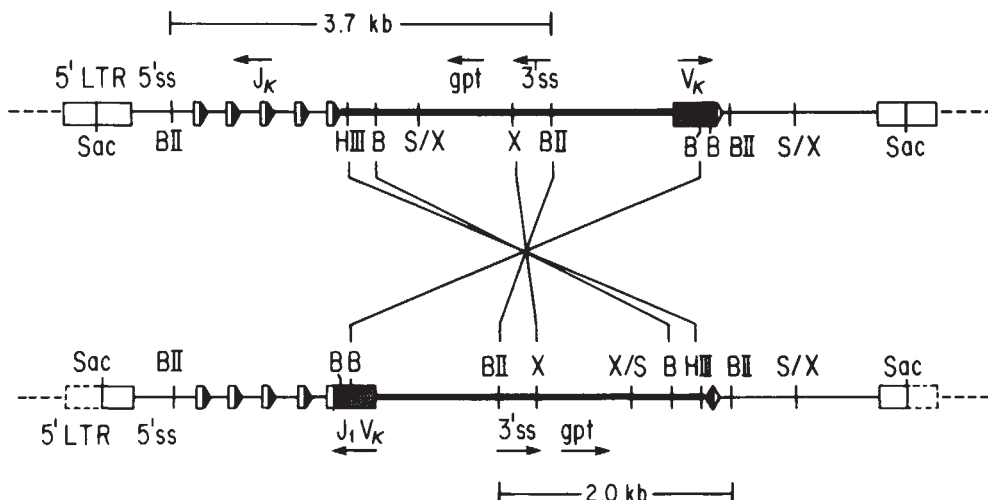
There were several advantages to using a retrovirus vector as a means of introducing the J_{κ} and V_{κ} segments into lymphoid cells. First, introduction of the sequences to be joined as a defective retrovirus simplified further analysis because retroviruses are generally integrated at the edges of their LTRs. This feature ensures that the substrate sequences will not be fortuitously interrupted or rearranged by their insertion into chromosomal DNA. Second, retrovirus infection is a more efficient and controlled process than DNA transfection. Third, the efficiency of introducing substrate could be maximized by using the ψ -2 packaging cell line because the virus stocks it produces are free of helper virus⁸.

The PD cell line which we chose to infect with the substrate virus is an Abelson murine leukaemia virus-transformed lymphoid cell line that can carry out V_{κ} - J_{κ} joining in culture⁴ and is superinfectible by Moloney MuLV. One to two days after the infection of 10^5 PD cells with substrate virus, the cells were placed in microtitre wells at $2-4 \times 10^3$ cells per well and were grown thereafter in the presence of $0.5 \mu\text{g ml}^{-1}$ mycophenolic acid to select clones expressing the *E. coli gpt* gene^{7,11}. At 3-4 weeks following plating, from one to five mycophenolic acid-resistant clones appeared per experiment and a number were isolated for further study. All but one of the isolates expressed the introduced bacterial enzyme by direct assay (ref. 11; data not shown) and the negative one was discarded.

Inversional joining

Mycophenolic acid-resistant PD clones in which the *gpt* gene was being expressed could have arisen either by inversional

Fig. 1 Maps of the substrate (top) and one predicted product of V_{κ} - J_{κ} joining (bottom). Top, partial map of the substrate, pVJG. The sources of the component fragments were as follows: the *Bgl*III-*Xho*I fragment bearing a 5' LTR, 5' splice site, pBR322 sequences, polyoma origin of replication and 3' LTR (of which only the ends of the fragment bearing the 5' LTR, 5' splice site and 3' LTR are represented in the figure) was derived from pMSVgpt⁷; the *Bgl*III-*Sac*I fragment including the J_{κ} cluster was derived from pHJ κ (ref. 4); the *Xho*I-*Bgl*III fragment containing the *gpt* gene and the 3' splice site was derived from a construct denoted pTRP-3'ss-MSVgpt (a gift of S. Hartman and R. Mulligan, unpublished) and the *Bgl*III-*Sac*I fragment containing the V_{κ} sequences was derived from Ig κ 5E (a gift from S. Tonegawa). The *Eco*RI-*Hind*III V_{κ} -containing fragment of Ig κ 5E was first subcloned into pBR322 (designated pV κ 14 and constructed by S. Desiderio) and then the unique *Eco*RI site was changed to a *Sac*I site. The rest of the construction was built in four stages, the details of which can be provided on request. Represented restriction endonuclease sites are abbreviated: Sac, *Sac*I; X, *Xho*I; BII, *Bgl*III; B, *Bam*HI. All the cleavage sites for these enzymes occurring between the *Sac*I sites in the LTRs of pVJG are indicated. Bottom, predicted structure of the region between the LTRs in pVJG if V_{κ} joined to J_{κ} 1 by inversion. This map also shows restriction sites found in a λ phage clone designated 2-1 and derived from cell line PD-A as described in the text. Clone 2-1 was isolated from a library of 5×10^5 recombinant λ phage bearing size-selected *Sac*I-cut DNA fragments from PD-A cell DNA.



V_{κ} - J_{κ} joining, so that the gene was expressed from the 5'-LTR promoter, or by the *gpt* gene acquiring an adventitious promoter from cellular sequences at the insertion site. (The retroviral construct was designed so that the *gpt* gene could be expressed via a spliced mRNA but we have not determined whether the expected spliced mRNA is produced.) The structure that would arise after inversional joining of V_{κ} to J_{κ} 1 is shown in Fig. 1 (bottom); it is evident that a new series of fragments should be detected by restriction enzyme cleavage at sites around the joints of an inverted segment. As one test of site-specific joining, DNA was prepared from a number of mycophenolic acid-resistant PD cell isolates, cut with *Bgl*III, and probed by hybridization to *gpt* gene sequences. In the original construct, the *gpt* gene occurred on a 3.7-kb *Bgl*III fragment (as indicated in Fig. 1, top). This fragment was detected in the virus-producing ψ -2 derivative (Fig. 3, lane ψ -2VJG), indicating that no major rearrangement of the substrate had occurred in the virus-producing cell line; the parental ψ -2 line had only the high molecular weight *gpt*-containing fragment introduced during its derivation (Fig. 3, lane ψ -2; see ref. 8. Rearrangement of the substrate in recipient PD cells should produce novel *Bgl*III fragments. The DNA from all nine mycophenolic acid-resistant PD clones assayed (representing at least seven independent isolates) showed an altered *Bgl*III fragment of 2 kb (Fig. 3). This *gpt*-containing fragment had the size that would be expected if V_{κ} 21-C joined to J_{κ} 1 by inversion (Fig. 1, bottom). Thus, it appeared likely that the *gpt* gene was being activated by the predicted inversional join and not by acquisition of adventitious promoters in the various mycophenolic acid-resistant PD cell lines.

Joint regions

An inversion forms two new DNA joints: in the present case it was expected that one would be a V_{κ} - J_{κ} coding joint and the other would be its reciprocal joint. In all lines assayed, the *Bgl*III digest implied that the reciprocal joint was derived from a recombination to J_{κ} 1, and not to any of the other J_{κ} genes. It seemed odd, however, that other DNA digests should indicate that the substrate in the nine PD isolates had formed a variety of coding joints, presumably via recombination to J genes other than J_{κ} 1 (the data will be presented in detail elsewhere). This apparent lack of reciprocity between coding joints and reciprocal joints, and further, this apparent over-representation of J_{κ} 1-derived reciprocal joints, is reminiscent of the situation in myelomas^{2,6}. We believe that secondary recombination, at high

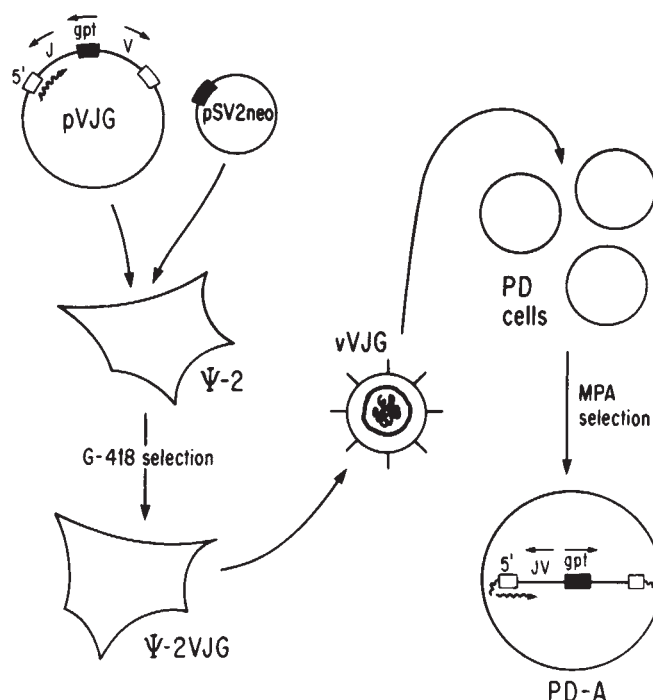


Fig. 2 Outline of the procedure used to introduce substrate sequences into the target cell line (PD): 10 μ g of pVJG and 1 μ g pSV2neo (ref. 19) were transfected into ψ -2 cells as described in ref. 8. Four *neo*-positive transfected ψ -2 cell lines were selected in medium containing 1 mg ml⁻¹ of the drug G-418 (Gibco)²⁰, then screened for the production of recombinant virus (vVJG). A virus-producing line was found among these four lines by demonstrating that medium from those cells could convert PD cells to mycophenolic acid resistance. For this assay, 1 ml of medium from the transfectants was applied to PD cells in the presence of 8 μ g ml⁻¹ polybrene for 1-2 h. One to two days post-infection, PD cells were plated directly in selective medium (RPMI 1640, 10% heat-inactivated fetal calf serum, 50 μ M 2-mercaptoethanol, 0.5 μ g ml⁻¹ mycophenolic acid (Lilly), 250 μ g ml⁻¹ xanthine, 15 μ g ml⁻¹ hypoxanthine and 150 μ g ml⁻¹ glutamine²¹) as described in the text. *gpt*⁺-Positive clones appeared in 3-4 weeks and were maintained in selective conditions.

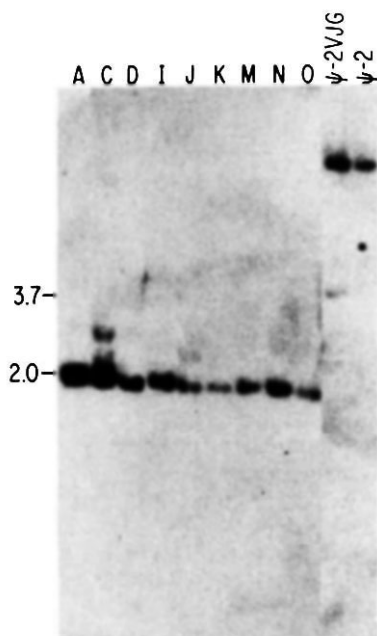


Fig. 3 Analysis of *Bgl*II-digested cellular DNA samples from mycophenolic acid-resistant PD cell lines. PD isolates A, C, D, I, J, K, M, N and O are shown, as is the fibroblast virus producer, ψ -2VJG, and its parental line, ψ -2. The filter was probed with *gpt* gene sequences, gel-purified from a *Hind*III, *Bam*HI digest of plasmid pL10 (a gift of R. Mulligan) which is a subclone of pPT-1¹¹.

frequency, deletes reciprocal joint regions down to fusions between the signal elements of V_{κ} and $J_{\kappa}1$ regardless of their original structure. Therefore, to simplify the present analysis, we selected a cell line, PD-A, for further study, on the basis that its DNA appeared to have a single proviral integrant that had undergone a single inversional join without secondary deletion. It was expected that in this case we might recover substrate sequences with both a coding joint and a reciprocal joint derived from $J_{\kappa}1$, and thus recover a pair of reciprocal recombination products.

Provirus sequence

To analyse the structure of the recombined substrate in more detail, the proviral sequences in PD-A were cloned into λ gt10 by digesting PD-A DNA with *Sac*I, an enzyme that cuts only in the LTRs of the substrate, and screening recombinant phage for the clone of interest with the *gpt* gene probe.

Restriction enzyme analysis of the clone we obtained indicated that it had the structure shown in Fig. 1 (bottom). The only alteration relative to the original construction appeared to be a large internal inversion, the end points of which suggested that a V_{κ} - J_{κ} recombination had occurred.

The DNA sequence at the inversional end points was determined using the method of Maxam and Gilbert¹². The precursor $V_{\kappa}21-C$ and $J_{\kappa}1$ sequences are shown in Fig. 4 together with the two recombinant joints. The sites at which the $V_{\kappa}21-C$ and $J_{\kappa}1$ elements were fused to form the coding joint can be unambiguously assigned and are indicated by arrows in the figure. The cross-over point on $V_{\kappa}21-C$ shown here is identical to that observed in the myeloma MOPC 321 (where the same *V* gene has fused with $J_{\kappa}2$; ref. 13). Similarly, the cross-over point we observed on $J_{\kappa}1$ is consistent with the site where $J_{\kappa}1$ recombined in another myeloma, MOPC 41⁵. In the present case, the reading frames of the fused V_{κ} and J_{κ} elements do not match, but out-of-frame joining of immunoglobulin gene components is well documented (for a review see ref. 14). Thus, the coding joint we observe has features that in all respects are consistent with its formation via the immunoglobulin gene recombination system.

Although the sequence of the coding joint was consistent with

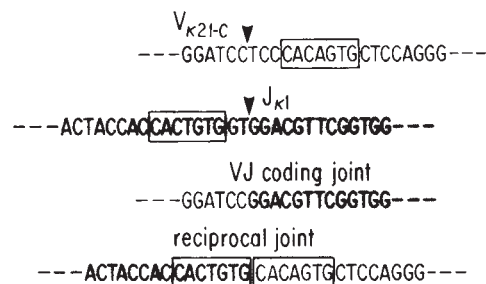


Fig. 4 Sequence of parental and recombinant fragments. $V_{\kappa}21-C$: 32 P end-labelled, *Bam*HI-digested DNA was prepared from pV $_{\kappa}14$ (see Fig. 1 legend) and recut with *Bgl*II. The fragment extending from the 3'-most *Bam*HI site in the coding portion of $V_{\kappa}21-C$ to the 3' proximal *Bgl*II site (see Fig. 1, upper) was electrophoretically purified for sequencing¹². The sequence could be read for 65 bases past the *Bam*HI site (not shown). $J_{\kappa}1$: The sequence shown is from a published report¹³. Coding joint: Clone 2-1 DNA was cut with *Bam*HI, 5'- 32 P-labelled with polynucleotide kinase and recut with *Bgl*II. The 3'-most *Bam*HI site in the coding portion of $V_{\kappa}21-C$ was labelled by this procedure, and the fragment extending from this *Bam*HI site across the coding joint to the *Bgl*II site 3' to the J_{κ} cluster was purified (see Fig. 1, bottom). The sequence was read for 63 bases past the recombinant joint and agrees completely with the published $J_{\kappa}1$ sequence¹³. Reciprocal joint: The *Hind*III-*Bgl*II fragment spanning the reciprocal joint of clone 2-1 (Fig. 1, bottom) was sequenced in both directions. The 50 bases read 3' to the joint were identical to the sequence obtained for the 3' flank of $V_{\kappa}21-C$ (not shown). The 70 bases read 5' to the joint were identical to the published sequence of the 5' flank of $J_{\kappa}1$ (ref. 13). Arrowheads indicate the positions of the recombination site used in coding joint formation relative to unrearranged $V_{\kappa}21-C$ and $J_{\kappa}1$ DNA sequences; the inside borders of the boxed sequences in $V_{\kappa}21-C$ and $J_{\kappa}1$ represent the position of the recombination site used in reciprocal joint formation.

one of several possible expected structures, an even more compelling indication that immunoglobulin-specific joining processes caused the inversion in PD-A was provided by the reciprocal joint. The reciprocal joint was an exact apposition of the heptamer recognition signals that were initially associated with $V_{\kappa}21-C$ and $J_{\kappa}1$ (Fig. 4). The analogous structure, a precise junction between heptamer elements, has been found in those reciprocal joints that have been cloned from lymphoid cells^{2,5,6,15}. Thus, these data clearly show that enzymes in the cell can recognize κ gene-derived segments on the substrate and rearrange them site-specifically. By extension, the identical size of the *Bgl*II fragment in the various PD derivatives expressing *gpt* (Fig. 3) implies that similar events activated the gene in all of the cells.

Mechanism of joining

Because both coding joints and reciprocal joints are found in many B-lymphoid tumour cells and because, as we show here, both of these joints can be generated by an inversion, the results described here support our previous proposal that in pre-B lymphocytes immunoglobulin gene joining may be accomplished through an inversional process⁴. The evidence that reciprocal joints in tumour cells are often not the direct reciprocals of the accompanying coding joints^{2,6,15} does not invalidate this suggestion because there is evidence from rearranging cells that the κ locus can undergo multiple recombinations⁴. Thus, reciprocal joints and coding joints, once formed, may be subjected to subsequent recombination which would serve to obscure their original relationship. Other proposals for the origin of reciprocal joints have been made^{2,3} but positive evidence for them is lacking. Because the distinction between our proposal and alternative schemes is basically topological, the question will probably remain open until more is known about the relative orientation of V_{κ} and J_{κ} in the murine germ line.

A direct conclusion obtained from the comparison of reciprocal recombination products and their precursor sequen-

ces (Fig. 4) is that joining can be accompanied by a net loss of a small number of nucleotides. Three nucleotides from the germ-line 3' flank of $V_{\kappa}21-C$ and two from the 5' side of $J_{\kappa}1$ were not found in either the coding joint or the reciprocal joint after recombination. Missing nucleotides have also been noted in a case where a non- V_{κ} -derived DNA segment recombined with $J_{\kappa}1$ (ref. 5) and was suggested to be a general feature of immunoglobulin gene joining processes^{6,15}. Nucleotides are lost because the cross-over site for the reciprocal joint is not positioned at the same base pair as the cross-over site for the coding joint (see Fig. 4). As mentioned earlier, although the cross-over site for reciprocal joints is invariant in all available examples (refs 2, 5, 6, 15 and this paper) there is evidence for considerable junctional diversity at coding joints¹⁶⁻¹⁸ which indirectly implies that nucleotides must be lost during a $V_{\kappa}-J_{\kappa}$ joining event.

A limitation of the present study is that the recombination substrate before rearrangement had no active selectable marker. Thus, it was impossible either to titre the substrate virus stocks directly or to determine the frequency with which recombination occurred in infected cells. It is also difficult to rule out the unlikely possibility that the observed rearrangements took place in the fibroblast ψ -2 producer line before the virus was used to infect PD, although mycophenolic acid resistance could not be passed from the ψ -2 cells to NIH 3T3 cells. We are presently constructing substrates that should allow quantitation of the frequency of this sort of recombination in a variety of cell types.

Conclusion

With the ability to examine the sequence of both products of a single joining event, we have been able to present evidence for

the first time that $V_{\kappa}-J_{\kappa}$ joining can occur via inversion, that it is a reciprocal exchange and that the joining enzymes act asymmetrically to unite heptamer elements at a fixed position while apparently eliminating bases from the coding joint. In addition, the effective rearrangement of the substrate implies that the joining reaction is not dependent on long-range chromatin or DNA structure, nor even on the presence of the constant-region portion of the κ gene. The ability to insert designed constructions into cells that will then undergo recombination should be very useful in further elucidating the mechanism, sequence requirements, and perhaps eventually the control of immunoglobulin gene joining.

In independent studies, K. Blackwell and F. Alt (personal communication) have observed rearrangement of heavy-chain D and J regions transfected into Abelson murine leukaemia virus-transformed fetal liver cells.

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Note added in proof: Cloning and DNA sequence analysis of additional independently-derived proviral recombinants confirm that $V_{\kappa}21-C$ in the substrate can recombine to J_{κ} segments other than $J_{\kappa}1$ and that in several examples of recombination to $J_{\kappa}1$ the resulting coding joint is variable. In contrast, all reciprocal joints analysed (including that found on a clone with a $V_{\kappa}-J_{\kappa}4$ coding joint) have a sequence identical to that in Fig. 4.

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A conserved DNA sequence in homoeotic genes of the *Drosophila* Antennapedia and bithorax complexes

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A repetitive DNA sequence has been identified in the Drosophila melanogaster genome that appears to be localized specifically within genes of the bithorax and Antennapedia complexes that are required for correct segmental development. Initially identified in cloned copies of the genes Antennapedia, Ultrabithorax and fushi tarazu, the sequence is also contained within two other DNA clones that have characteristics strongly suggesting that they derive from other homoeotic genes.

MANY of the homoeotic genes of *Drosophila* seem to be involved in the specification of developmental pathways for the body segments of the fly, so that each segment acquires a unique identity. A mutation in such a homoeotic gene often results in a replacement of one body segment (or part of a segment) by

another segment that is normally located elsewhere. Many of these homoeotic loci reside in two gene complexes, the bithorax complex and the Antennapedia (Antp) complex, both located on the right arm of chromosome 3 (3R).

The bithorax complex is located in the middle of 3R, and its resident genes impose specific segmental identities on the posterior thoracic and abdominal segments¹. For example, inactivation of the *bithorax* gene of the complex causes a transformation

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of the anterior half of the third thoracic segment into the anterior half of the second thoracic segment, resulting in a fly having wing structures in a site normally occupied by haltere. Other recessive mutations in the complex cause analogous transformations of posterior body structures into structures normally located in a more anterior position. Embryos having a deletion of the entire bithorax complex show a transformation of all the posterior body segments into reiterated segments with structures of the second thoracic segment. Based on the above results and others, Lewis has proposed a model in which segmental identity in the thorax and abdomen is controlled by a stepwise activation of additional bithorax complex genes in more posterior segments¹.

The *Antp* complex is localized nearer the centromere of 3R than the bithorax complex. The genes of the *Antp* complex appear to control segmental development in the posterior head and thorax, in a manner analogous to the way in which the bithorax complex operates in the more posterior segments²⁻⁵. A dominant mutation in the *Antp* locus, for example, can result in the transformation of the antenna of the fly into a second thoracic leg^{6,7}.

The homoeotic genes of both the bithorax and *Antp* complexes can be thought of as selector genes, using the nomenclature of Garcia-Bellido⁸, that act by interpreting gradients of positional information. Based on their location in the gradient, a specific combination of selector genes are expressed, and thus different regions of the developing fly become selected to proceed down specific developmental pathways. Although the available evidence supports this model^{1,9,10}, the real situation appears to be more complex as there is also evidence that regulatory interactions between different homoeotic selector genes have a role in limiting their region of expression¹⁰⁻¹².

The physical proximity and similar but distinct functions of the bithorax complex genes led Lewis to propose that the genes of this cluster evolved by mutational diversification of tandemly repeated genes¹. In the primitive millipede-like ancestors of *Drosophila*, an ancestral gene or genes would direct the development of repetitive segments having similar identities. The evolutionary transition to the Dipterans, with highly diverse segmental structures, might be achieved by duplication and divergence of ancestral genes. According to this model, null mutations in the present set of bithorax complex genes could result in a fly having a more primitive segmental array, that is, with legs on the abdominal segments, or with wings on the third thoracic segment, in addition to those on the second thoracic segment; both types of phenotype are known to result from reduction or loss of function of bithorax complex genes.

Although the bithorax and *Antp* complexes are widely separated on the third chromosome, their similar functions in specifying segmental identity suggests that both complexes might have evolved from a common ancestral gene or gene complex. A critical test for this hypothesis involves a test for conserved sequences in the genes of the two complexes. These conserved sequences could be relics of ancient gene duplications or regions specifically preserved by selection against mutational change. Here we show that there is DNA sequence homology between some genes of the bithorax complex and the *Antp* complex. We use this homology, which is imperfect and limited to small regions, to isolate other cross-hybridizing clones from the *Drosophila* genome. The cytogenetic map locations and spatial and temporal patterns of expression for the genes homologous to two of the clones suggest that they represent other homoeotic genes.

Repeated sequences

Genomic and cDNA clones from the *Antp* locus have been isolated and characterized by Garber *et al.*¹³. To test whether the *Antp* gene might be a member of a multigene family, we hybridized the 903 cDNA probe derived from the *Antp* locus to Southern blots of *Drosophila* genomic DNA. The 903 cDNA (see Fig. 1) is complementary to four non-contiguous chromosomal DNA regions spanning 100 kilobases (kb) at the *Antp*

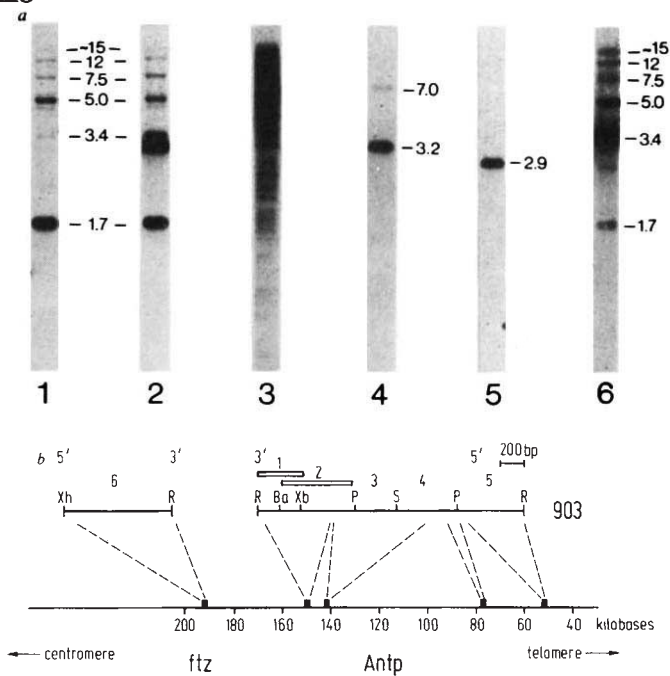


Fig. 1 Repeated sequences in the *Antp* and *ftz* genes. *a*, Individual lanes from *Drosophila* whole genomic Southern blots. The genomic DNA in each case was digested with *EcoRI*. The number below the lanes designates the fragment number used as a probe. The fragment number designations are shown above the respective fragments in *b*. The numbers alongside the lanes indicate the sizes in kilobases (kb) of the hybridizing genomic fragments. All the blots were hybridized and washed in the reduced-stringency conditions described below. Note that the two bands in lane 4 are due to the number 4 probe containing a sequence from each of two *Antp* exons. *b*, Map of the portions of the *Antp* and *ftz* genes used as hybridization probes for the genomic blots. *Antp* 903 is a cDNA clone described by Garber *et al.*¹³ which contains the regions from the *Antp* locus marked by solid blocks. The broken lines indicate the approximate extent of the cDNA in each genomic location. The bottom line is a representation of the *Antp* region of chromosome 3, as taken from Garber *et al.*¹³; the numbers reflect the distance (in kb) from the *Humeral* chromosomal breakpoint. The 5' and 3' labels show the direction of transcription for the two loci (ref. 17 and A.K., unpublished results). Xh, *XhoI*; R, *EcoRI*; Ba, *BamHI*; Xb, *XbaI*; P, *PvuII*; S, *SphI*.

Methods: Reduced-stringency hybridizations were done as follows. Southern blots³¹ were prehybridized in 5×SSC, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 250 µg ml⁻¹ sonicated, boiled herring sperm DNA, 50 mM NaPO₄ pH 7, 0.1% SDS, 43% deionized formamide at 37 °C for 2–3 h. The prehybridization buffer was removed from the bag and replaced with the same buffer containing 10⁶ c.p.m. ml⁻¹ of hybridization probe. Blots were hybridized at 37 °C for 25–48 h, then washed twice in 2×SSC, 0.1% SDS for 5 min at room temperature, followed by two washes for 15 min each at 45–50 °C. Stringent hybridization and wash conditions differed only in the hybridization buffer, which contained 50% instead of 43% formamide, and in the final wash which was done in 0.2×SSC, 0.1% SDS at 65–70 °C.

locus. Both normal- and reduced-stringency hybridization conditions were used with the 903 probe; in both types of hybridization conditions we detected many genomic fragments homologous to 903 that gave very strong signals, and many (>50) that were relatively weak. The weak signals were more prominent on the blot hybridized in reduced-stringency conditions (data not shown). A stringent wash of both blots (see Fig. 1 legend) removed the weak signals whereas the strong signals remained. The strongly hybridizing genomic fragments had the expected size for those portions of the genome represented in the 903 cDNA. The weakly hybridizing genomic fragments presumably possessed mismatched homology to one or more repeated sequences within the 903 cDNA.

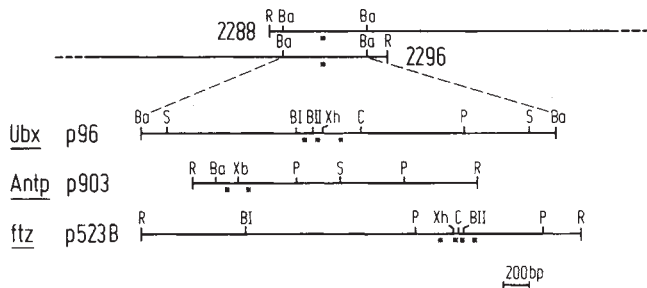


Fig. 2 Localization of the H repeat in clones from the *Ultrabithorax* (*Ubx*), *Antennapedia* (*Antp*) and *fushi tarazu* (*ftz*) genes. The overlapping region from the *Ubx* locus between λ clones 2288 and 2296 (gifts from P. Spierer) is shown at the top, as well as the *Bam*HI fragment common to both which hybridizes to fragments 2 and 6 (Fig. 1). Both these clones are described elsewhere¹⁵, although λ 2296 is incorrectly labelled as 2269. The indicated *Bam*HI fragment was subcloned into pAT153 and the resulting plasmid designated p96. A map of the p96 insert is shown. Restriction fragments containing the H repeat are marked by asterisks. *Antp* p903 is the same cDNA clone shown in Fig. 1. Restriction fragments to both sides of the *Xba*I site (Xb) show homology to the H repeat. *ftz* p523B is a *Drosophila* genomic clone from region 190 on the map in Fig. 1, an *Eco*RI fragment in pAT153. This fragment contains most of the *ftz* transcription unit (A.K., unpublished results). Again, the restriction fragments containing the H repeat are marked by asterisks.

To determine which region(s) within the 903 cDNA sequence were repetitive, the cDNA was subdivided into five restriction fragments of ~500 base pairs (bp) each, and these were individually hybridized to replica genomic Southern blots in reduced-stringency conditions (Fig. 1). The two left-most fragments, which overlap in the 3' half of the 903 cDNA, detect the expected genomic fragments at *Antp*, and also cross-hybridize with seven other genomic fragments with less intensity. The next 903 fragment to the right (fragment 3) hybridizes to more than 50 genomic fragments. Finally, the two right-most 903 fragments (4 and 5) detect only their genomic homologues at the *Antp* locus.

Garber *et al.*¹³ found weak homology between the 903 cDNA and a site to the left of the *Antp* locus, at position 190 on the map in Fig. 1. This site has subsequently been shown to be part of the transcription unit of the *fushi tarazu* (*ftz*) gene (A.K. and E.H., in preparation). The *ftz* gene is required for the determination of the correct number of segments in the *Drosophila* embryo^{4,14}. Embryos that are homozygous for certain mutant alleles of *ftz* die early in development, and show deletions of alternate segment primordia. A 0.9-kb *Xho*I/*Eco*RI fragment (probe 6), containing a 3' portion of the *ftz* transcription unit (A.K., unpublished results), was used as a probe of another Southern blot identical to those used for the five 903 fragments (Fig. 1). In addition to the strong signal contributed by the homologous genomic fragment from the *ftz* locus, eight other genomic fragments weakly cross-hybridized. Five of these weakly hybridizing genomic DNA fragments are identical in size to those detected by the two probes from the 3' region of the 903 cDNA. Thus, the 3' regions of the transcription units of the *Antp* and *ftz* genes share a common sequence, one that appears to be present at five or more locations in the *Drosophila* genome.

Subsequently, we will refer to this low-level repeat as the H repeat, and the high-level repeat in the middle of the 903 cDNA (fragment 3) as the M repeat. The M repeat is not detectable in the DNA of the *ftz* locus.

Presence of H repeat in bithorax complex

Next, we performed experiments to test for the presence of the H repeat in other homoeotic genes. We hybridized fragments 2 and 6 (from *Antp* and *ftz* respectively; see Fig. 1) in reduced-stringency conditions to Southern blots of recombinant clones from the *Ultrabithorax* (*Ubx*) unit of the bithorax complex,

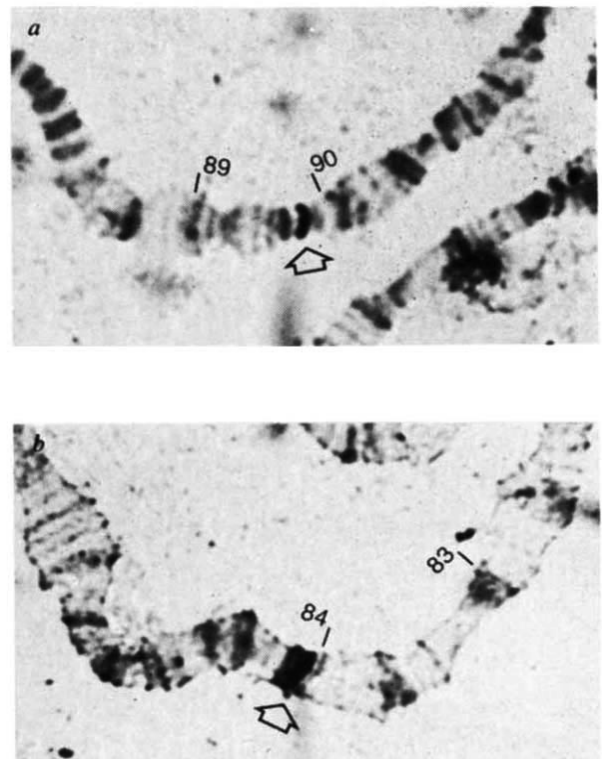


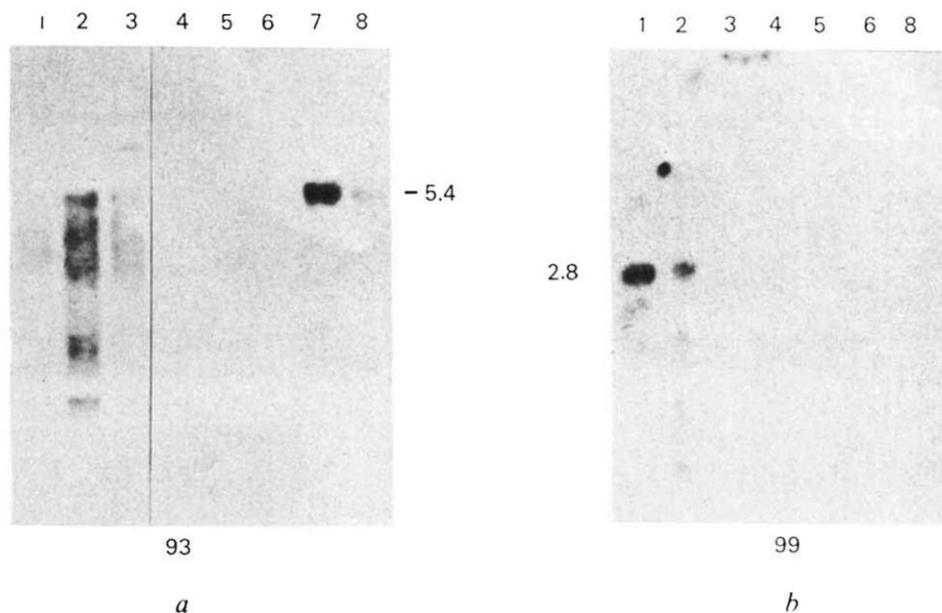
Fig. 3 *In situ* hybridization of H repeat clones p93 and p99 to polytene chromosomes. Clones p93 and p99 were nick-translated with biotinylated nucleotide, and hybridized to squashes of Ore-R chromosomes: p93 to a, and p99 to b. The hybridized probes were detected by an immunoperoxidase method²¹ and the chromosomes stained with Giemsa and photographed. The chromosome 3 divisions are indicated and the sites of hybridization marked by arrowheads.

specifically λ 2229, λ 2269, λ 2288 and λ 2296 (ref. 15) (given by P. Spierer). A 3.2-kb *Bam*HI fragment common to λ 2288 and λ 2296 hybridized to both H repeat-containing probes (Fig. 2). This *Bam*HI fragment contains most or all of the 3' exon of the *Ubx* transcription unit (refs 15, 16 and M. Goldschmidt-Clermont, personal communication). Cross-hybridization between the *Antp*, *ftz* and *Ubx* loci has been independently detected by M. Scott (personal communication). None of the *Ubx* region clones that we tested contained the M repeat.

Figure 2 shows more detailed restriction maps of the regions at each locus that contain the H repeat. The location of the H repeat within each of the *Antp*, *Ubx* and *ftz* clones was determined by *inter se* hybridizations to Southern blots of each clone digested with various restriction enzymes. The H repeat is the only detectable region of cross-homology between the three clones, and based on the intensity of signal the cross-homology is either very short (<100 bp), or larger but poorly matched. As the region of cross-homology overlaps both ends of the 90-bp *Xho*I/*Bgl*II fragments found in both *Ubx* and *ftz*, the latter possibility is more likely. The intensity of signal obtained from homologous hybridization compared with cross-hybridization due to the H repeat is shown in lane 1 of Fig. 1. Probe fragment 1, from the 3' end of the *Antp* cDNA 903, hybridizes very strongly to its genomic homologue on a 1.7-kb *Eco*RI fragment, but with at least 10 times less intensity to the 3.4-kb and 7.5-kb genomic fragments in the same lane, which carry the *ftz* and *Ubx* H repeats respectively.

The cloned regions in Fig. 2 are shown with the same 3R chromosomal orientation: the centromere is to the left, and the telomere to the right. The transcriptional direction of the *Ubx* and *Antp* clones is from right to left, and for the *ftz* clone from left to right (refs 16, 17 and A.K., unpublished results). The

Fig. 4 Transcription from p93 and p99 during *Drosophila* development. Lanes 1, 2 and 3 in each panel contain embryonic RNA from 0–6, 6–12 and 12–18-h stages, respectively. Lanes 4 and 5 in each panel contain RNA from first and second instar larvae. Lanes 6 and 7 in each panel contain RNA from early and late third instar larvae, respectively. Lane 8 in each panel contains RNA from 1-day-old pupae. The numbers alongside the panels indicate the approximate size (in kb) of the largest hybridizing RNAs. On longer exposures a faint band of 2.8 kb was detected in the pupal lane (8) of the blot hybridized with p99 (not shown). Poly(A)⁺ (10 µg) from successive stages of *Drosophila* development was run on formaldehyde agarose gels and blotted³². The blots were hybridized with nick-translated p93 and p99 in the stringent buffer described in Fig. 1 legend. After a stringent wash (also described in Fig. 1) the blots were used to expose X-ray film.



relative orientation of the *Xho*I and *Bgl*II sites within the cross-homologous regions of *Ubx* and *ftz*, is consistent with the polarity of the H repeat being the same with respect to the direction of transcription. Preliminary DNA sequencing results from these two regions (W. McG., unpublished results) and of the *Antp* 903 cDNA (R. Garber, unpublished results) also indicate that the H repeat is in the same orientation with respect to transcription at each locus.

Isolation of clones containing H repeat

The observation that the H repeat was associated with three loci known to be crucial for proper segmental development in the fly suggested that it might be a common feature of many homoeotic loci in *Drosophila*. To test this, we first isolated *Drosophila* genomic clones that possessed homology to cloned H repeat sequences. Such clones were then subjected to the following three tests to implicate them as potential new homoeotic loci.

(1) Hybridization of the clones to *Drosophila* polytene chromosomes to determine whether their cytogenetic map locations corresponded to those of genetically characterized homoeotic loci.

(2) Hybridization of subclones containing the H repeat to Northern blots of RNA extracted from successive developmental stages to determine whether the regions were transcribed, and whether their transcription was developmentally regulated in a manner that might be expected for a homoeotic locus.

(3) Hybridization of subclones containing the H repeat to *Drosophila* embryonic tissue sections, to determine whether the transcripts homologous to the subcloned regions were distributed in a segmentally restricted manner, as has been shown for transcripts derived from the homoeotic loci *Antp* and *Ubx*^{16,18,19}.

Genomic library screen

Approximately 30,000 recombinant bacteriophages (three genome equivalents) from the Charon 4/*Drosophila* library of Maniatis *et al.*²⁰ were screened using H repeat probes from *Antp* and *ftz* (fragments 2 and 6 in Fig. 1) as probes of duplicate filters. The hybridizations and washes were done in the reduced-stringency conditions described in Fig. 1 legend. A total of 74 plaques from the original plates hybridized to both probes, with a wide range of signal intensity. All were picked and re-screened with the same probes, and 24 were plaque-purified. DNA was

extracted from each recombinant and digested with *Eco*RI, *Bam*HI and both enzymes together, then separated on an agarose gel, and transferred to nitrocellulose.

These blots were hybridized with probes 2 and 6 to determine which region of the insert contained the H repeat. In this way, we found that three of the clones were re-isolates of *ftz*, and two were re-isolates of *Antp*. The remaining 19 clones were divided into two classes on the basis of their extent of homology (as determined by signal intensity) with the H repeats from *Antp* and *ftz*. Seven clones cross-hybridized relatively strongly and by *inter se* hybridizations we found that these were derived from two different genomic regions. Representative clones from each of the two regions were designated 93 and 99.

The H repeat lies on a 4.5-kb *Bam*HI/*Eco*RI fragment for clone 93; this fragment was subcloned into plasmid vector pAT153 and is designated p93. The p93 insert is a single-copy sequence in the *Drosophila* genome, when tested by genomic Southern blot analysis in stringent hybridization and wash criteria, and derives from the 15-kb genomic *Eco*RI fragment previously identified by genomic blotting (Fig. 1, lane 1). The clone 99 H repeat lies on a 5-kb *Eco*RI fragment which was cloned into pAT153 and designated p99. The p99 insert is also a single-copy *Drosophila* sequence (in stringent conditions), and corresponds to the 5-kb *Eco*RI genomic fragment detected in Fig. 1, lane 1. Although p99 and p93 were selected by hybridization with the H repeat they also hybridize with the probe 3 shown in Fig. 1, and therefore probably contain the M repeat also.

In situ localization of p93 and p99

DNA from p93 and from p99 was labelled with biotinylated dUTP by nick-translation, and hybridized to squashes of salivary gland polytene chromosomes. The sites of hybridization were revealed by an immunoperoxidase detection protocol²¹. The stringency of hybridization was sufficient to allow only the detection of the genomic isologues of p93 and p99.

Clone p93 hybridizes to the 89E region on the right arm of chromosome 3 (Fig. 3), the cytogenetic location of the bithorax complex¹. Bender *et al.*^{15,22} have recently reported the isolation of the left half of the bithorax complex DNA, and Karch and Bender have subsequently isolated overlapping cloned genomic DNAs that include much of the right part of the complex (F. Karch and W. Bender, unpublished results); they provided us with a Southern blot containing cloned DNA from the entire cloned bithorax region. We find that the p93 insert is located

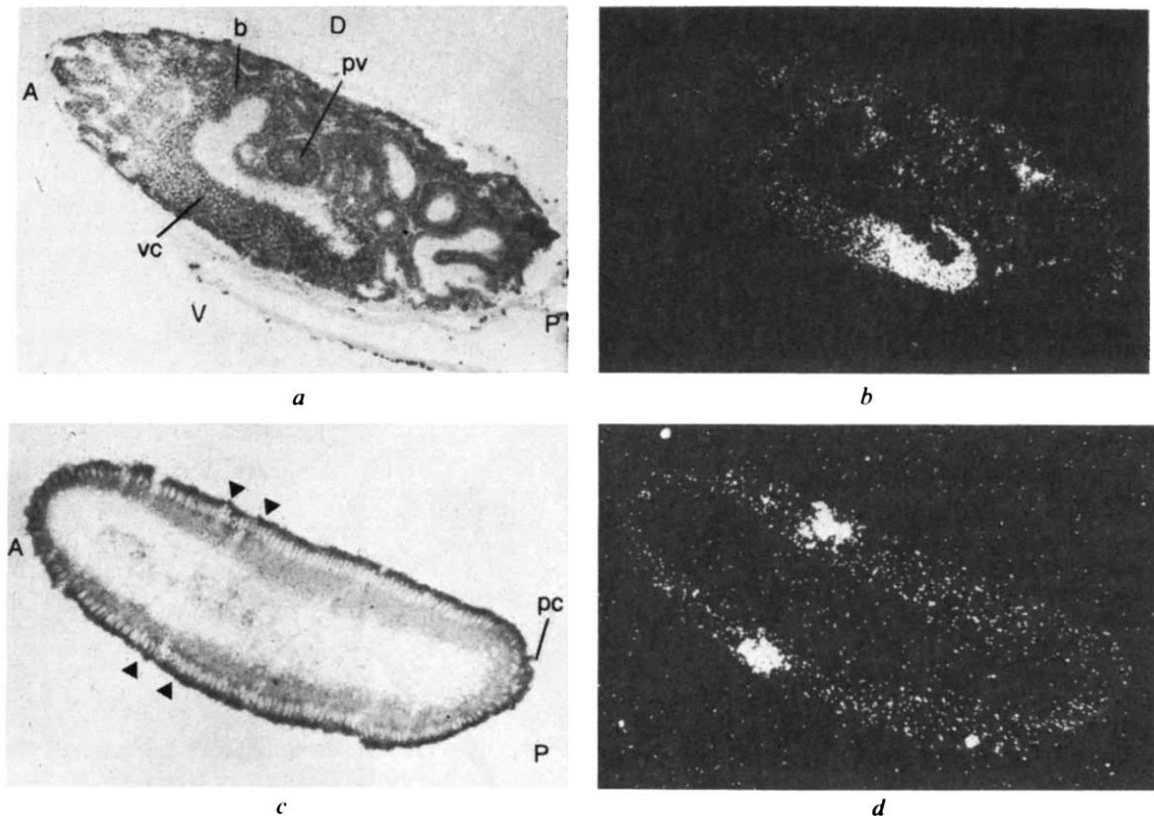


Fig. 5 Localization of transcripts homologous to p93 (a, b) and p99 (c, d) in embryonic tissue sections. The embryonic tissue sections were prepared and hybridized to ^3H -labelled probes as described by Hafen *et al.*¹⁸. Labelling of p93 is shown on a sagittal section of an 18–20-h embryo. Both brightfield (a) and darkfield (b) photographs of a 4-week autoradiographic exposure are shown. The p99 labelling is shown for an embryo 3 h old, just after the stage during which cell membranes envelop the nuclei at the periphery of the embryo. Both bright- and darkfield photomicrographs of a 4-week autoradiographic exposure are shown. The extent of hybridization of the brightfield view (a) is marked by arrowheads. A, anterior; P, posterior; vc, ventral nerve cord; b, brain; pv, proventriculus; pc, pole cells; D, dorsal; V, ventral.

within their cloned region, to the right of the *bithoraxoid/post-bithorax* unit of the bithorax complex, near the *infra-abdominal-2* locus (data not shown). The same result has been obtained by S. Sakonju (personal communication) and M.S.L.

Clone p99 hybridizes to the 84A region on the right arm of chromosome 3; this is close to the cytogenetic locations of the *ftz* and *Antp* loci, both of which are in 84B1-2 (refs 2, 4, 5). The 84A region contains the *Antp* complex genes *proboscipedia*, *zerknüllt* and *Deformed*, all of which have been shown to affect the proper development of the posterior head segments of *Drosophila*^{4,5,23}.

Transcription

Genetic and developmental studies on the time of expression of homoeotic selector genes show that early to mid-embryogenesis (0–12 h after oviposition) and pre- and early metamorphosis (late third instar larval and early pupal stages) are possible periods of high levels of expression^{24–26}. *Antp* transcripts exhibit their highest levels during both these periods, and *ftz* transcripts are most abundant in early embryogenesis (A.K., unpublished results).

To test whether the regions homologous to p93 and p99 were transcribed, the two clones were hybridized to Northern blots containing *Drosophila* poly(A)⁺ RNA from successive stages of development. The blots were hybridized and washed using the stringent conditions described in Fig. 1 legend.

Clone 93 is homologous to multiple RNA species during embryonic stages, especially in embryos 6–12 h old (Fig. 4). The largest RNA (5.4 kb) homologous to p93 at the 6–12-h stage is also abundant in late third instar larvae, just before pupation. Clone p99 is homologous to an RNA species of 2.8 kb which is most abundant at early embryogenesis (0–6 h) and in the early pupal stage (Fig. 4). The RNA species homologous to p93 and

p99 are both present at approximately the same levels as transcripts from the *Antp* locus (A.K., unpublished results).

Localization of transcripts

The most important experiment that we performed with the p93 and p99 clones was to test whether the transcripts homologous to these clones were spatially restricted during development. It has recently been shown that *Antp* and *Ubx* transcripts are restricted in a segmentally specific manner during embryonic development^{16,18,19}. To a rough approximation, the embryonic segments and segmental anlagen that accumulate *Antp* and *Ubx* transcripts are those in which the function of these genes is believed to be required for proper development. Therefore, if the 93 and 99 clones represent other homoeotic selector loci, their transcripts should be restricted during embryonic stages to segments where 93 or 99 expression is required for proper development.

Figure 5 shows that the transcripts homologous to both p93 and p99 show spatial restrictions during development. We have shown only one developmental stage, when transcript localization is striking, for each clone. More detailed studies on the expression of these cloned regions will be reported elsewhere.

The localization of transcripts homologous to p93 is shown on a sagittal section of an 18–20-h embryo. At this advanced stage of embryogenesis, the central nervous system includes the two brain hemispheres and the condensed ventral nerve cord (Fig. 5). Before condensation the ventral cord consists of 12 paired ganglia, the sub-oesophageal ganglion, three thoracic ganglia and eight abdominal ganglia. Each ganglion of the ventral cord innervates its corresponding body segment²⁷. The entire central nervous system of the embryo section hybridized with p93 appeared to be labelled above background levels, but a striking and reproducible concentration of label was observed

over the posterior region of the ventral nerve cord, encompassing at least the posterior-most five or six abdominal neuromeres.

The labelling pattern of p99 is shown at the cellular blastoderm stage, ~3 h after oviposition. The cellular blastoderm consists of a monolayer of morphologically identical cells. It is at this stage that cells first become restricted in their developmental potential, with different regions of the blastoderm acquiring separate determinative fates^{28,29}. Transcripts homologous to p99 were found to be concentrated in cells in about 60–65% of the embryo length from the posterior pole. Cell ablation experiments in this region of the cellular blastoderm result in embryos having defects in the first thoracic and posterior head segments³⁰.

Conclusions

Our analyses of the 93 and 99 clones, both isolated with the H repeat cross-homology, strongly suggest that they represent other homoeotic loci of *Drosophila*. Both clones fulfilled all three criteria that we applied for representing clones from homoeotic loci. First, both hybridize to cytogenetic locations of previously characterized homoeotic genes; 93 to the right half of the bithorax complex in the chromosome region 89E, and 99 to chromosome region 84A, which contains genes in the proximal half of the Antp complex. Second, both 93 and 99 are homologous to transcripts that are relatively abundant during embryogenesis and just prior to metamorphosis. These are the periods when transcripts homologous to the homoeotic locus *Antp* are most abundant (A.K., unpublished results). Third, and most importantly, the transcripts homologous to 93 and 99 show a striking spatial restriction during development. Transcripts homologous to p93 are most abundant in the posterior abdominal neuromeres of the embryo, as would be expected from a gene in the right half of the bithorax complex. The transcripts homologous to p99 are most abundant in a region of the cellular blastoderm that corresponds to the segmental anlagen of the posterior head or first thoracic segments. This is also consistent with its cytogenetic location in 84A, which contains genes that affect the development of these segments.

The basis for the cross-homology is of great interest. The position of the H repeat in the 3' region of the transcription units of *Antp*, *Ubx* and *ftz* is consistent with a conserved protein-coding sequence. The DNA sequence of the H repeats of *Antp*, *ftz* and *Ubx* leaves no doubt that the sequence conservation is due to a conserved protein-coding domain (W.McG. and R. Garber, unpublished results). Since faithful copies of the H repeat are strictly delimited and found only in homoeotic genes, we now call the H repeat the 'homoeotic sequence'. However, it seems clear that not all homoeotic genes carry the homoeobox, for example, we have been unable to detect it in the *bithoraxoid*/*postbithorax* unit of the bithorax complex (W.McG., F. Karch and W. Bender, unpublished results). It is possible, of course, that another subset of homoeotic genes contains another repeat.

On the basis of these results, we propose that a subset of the homoeotic genes are members of a multigene family, highly diverged but nonetheless detectable by DNA cross-homology. This suggests a common evolutionary origin for some genes of both the Antp and bithorax complexes, as proposed by Lewis¹ for the genes of the bithorax complex. The conspicuous evolutionary conservation of the homoeobox sequence in some homoeotic genes of *Drosophila* suggests that it might also be conserved in other animal species; preliminary experiments strongly support this view (W.McG., unpublished results). It is possible that a fundamental principle in development is to duplicate a gene specifying a segment identity, allowing one of the copies to diverge and acquire new functions, or new spatial restrictions in expression, or both; this might allow, within the limits of natural selection, a striking polymorphism in the different segments of an animal, and the acquisition of highly specialized functions in different segments.

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Frequency of fast, narrow γ -ray bursts

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Early studies of the time histories of the γ -ray burst events reported by the Vela satellites led to the suggestion that very brief, ~ 0.1 s, bursts formed a class distinct from the longer, highly structured bursts¹. Results of the KONUS experiments on Veneras 11, 12, 13 and 14 argue for the existence of a separate class of short-duration γ -ray bursts², revealing that many of the short bursts exhibit much softer spectra (~ 30 keV) than the more familiar complex bursts. Although this correlation is not completely clear and the observations are in some cases contradictory²⁻⁶, the existence of two γ -ray burst populations is suggested by Fig. 1, showing a duration histogram for 24 events detected by our ISEE-3 experiment. Figure 1 also shows the distribution of 123 Venera 13 and 14 events (60 detected by both spacecraft) and suggests two γ -ray burst populations in each experiment sample, the domains separated with a minimum near 1 or 2 s. Here we point out that the results of the Goddard ISEE-3 γ -ray burst spectrometer actually enhance the appearance of two burst populations suggested in the Venera data.

The ISEE-3 burst spectrometer^{7,8} trigger criterion differs from previous techniques in that the time for detecting a preset number of photons is accumulated ('time to spill'), rather than accumulating counts over a preset time interval. The photon counter setting and timing precision (clock frequency) are adjustable by factors of two, within limits. The most often used values have been 32 counts and 1,024 Hz. This method has the advantage of recording the more intense portions of the time history with greater temporal precision (the Los Alamos experiments aboard Pioneer Venus Orbiter and ISEE-3 utilize the

time-to-spill mode only for recording the fine-time structure of intense intervals of bursts but do not utilize time-to-spill as the event trigger criterion). The trigger technique also provides a basic new advantage. The conventional detection problem of discriminating against bursts with durations less than the trigger integration time is avoided. Consequently, although the ISEE-3 experiment is not one of the most sensitive γ -ray burst sensors, the ISEE-3 event sample may be more representative in that it contains a truer proportion of brief events. Since spectral information from our experiment is no longer available, burst fluences cannot be accurately determined. However, if a hard spectrum characteristic of 'classical' γ -ray bursts is assumed, the estimated ISEE-3 detection threshold is $\sim 10^{-5}$ erg cm⁻² s⁻¹.

Nine out of twenty-four cosmic events detected by the ISEE-3 experiment from September 1978 to May 1982 exhibited durations less than one second, risetimes of tens of milliseconds, and usually no discernible substructure other than an exponential decay in some cases. The 5 March 1979 event⁹, which exhibited an intense initial pulse of FWHM ~ 120 ms (risetime < 200 μ s), is included in this group since the unique eight-second oscillating decay phase would not have been discernible had the intensity of the initial pulse been comparable to flux levels ordinarily observed in γ -ray bursts. The temporal characteristics of the ISEE-3 short events are summarized in Table 1. Since the count rate data are generated using the time-to-spill technique, for a given detector area, the observed risetimes and widths are a function of the photon counter setting, the burst intensity, and the intrinsic temporal characteristics. Therefore, for low intensity bursts, the temporal resolution is poor and the observed risetimes and widths are probably somewhat longer than the intrinsic times.

Six out of nine of the short ISEE-3 bursts have been confirmed to be of cosmic origin by other spacecraft. For three reasons the remaining events are believed to be genuine γ -ray burst detections. First, for two of the unconfirmed events, one would need statistical fluctuations, in order to equal or exceed the burst peaks, that would be expected once in ≥ 27 years, much longer than the experiment lifetime. Second, in all three unconfirmed events, the duration was ≤ 0.1 s. This could possibly explain why the bursts remained undetected by other experiments. Third, the germanium detector, which detected all of the short bursts, is not sensitive to charged particles which simulate spike-like events in caesium iodide scintillators. Since the KONUS 13/14 distribution of short events continues down to ~ 0.1 s (Fig. 1), the ISEE-3 unconfirmed events probably do not represent a separate class of very short bursts.

The ISEE-3 result has been recently substantiated by an increase in the frequency of detection of short bursts in the KONUS 13/14 data base over KONUS 11/12. The KONUS experiments flown on the earlier Venera 11 and 12 spacecraft (see ref. 10) utilized trigger mechanisms with two integration time constants of 0.3 s and 1.5 s, the shorter included to facilitate the detection of fast events. As reported by E. P. Mazets

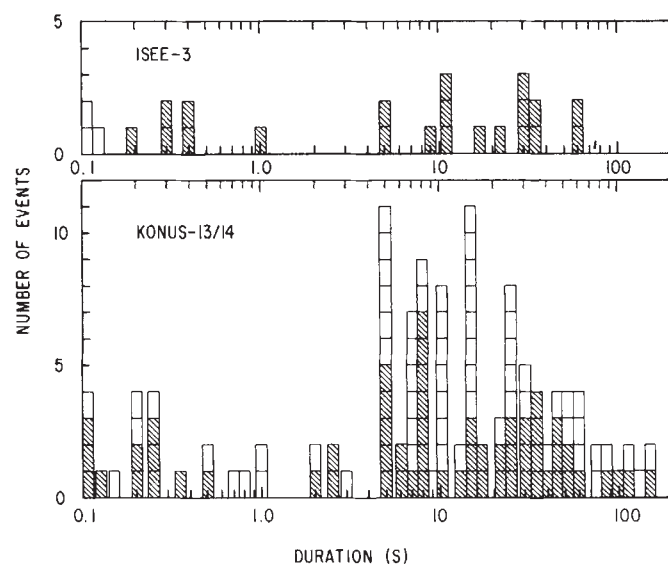


Fig. 1 Distribution of γ -ray burst durations for ISEE-3 and KONUS-13/14. The shaded areas represent confirmed events, unshaded areas for unconfirmed (one spacecraft only) events. Repeated bursts from the 5 March 1979 source (B0250-66) are not included in the KONUS distribution.

Table 1 ISEE-3 short events

Event	Peak (c.p.s.)	Risetime (s)	FWHM (s)	FWZA (s)
5 March 1979	32,500	< 0.0002	0.12	0.30
15 March 1979	520	0.30	< 0.06	0.06
12 May 1979	420	0.04	< 0.08	0.08
22 March 1980	360	0.05	0.36	1.0
6 August 1980	431	0.06	0.17	0.44
5 December 1980	607	0.04	0.05	0.19
31 December 1981	262	0.02	< 0.30	0.30
8 May 1982	278	0.05	< 0.10	0.10
19 May 1982	910	0.01	0.07	0.40

FWHM, full-width at half-maximum intensity. FWZA, full-width at zero-amplitude response.

(personal communication), the detector sensitivity is strongly dependent on the event duration. Speaking in terms of flux, the more interesting quantity from a source population viewpoint, rather than fluence, the threshold for detection increases with decreasing burst duration: the KONUS detection threshold for bursts of duration less than the trigger integration time is approximately inversely proportional to the duration. The instrumental configurations for the Venera 13 and 14 experiments were similar to the previous KONUS system¹¹. However, the short integration time constant was set at 0.25 s rather than 0.3 s. On inspection of the Venera 11/12 and 13/14 data bases, we find that the change in trigger time constants is apparently reflected in a change in the frequency of detection of short bursts. For KONUS 11/12, the ratio of short bursts to total bursts is 0.07 ± 0.02 , whereas for KONUS 13/14, a much higher percentage is indicated, 0.16 ± 0.04 . These figures do not include repeated bursts from the sources B1900 +14 and B0520 -66^{2,11}. If only the confirmed ISEE-3 events are considered, the ratio of short bursts to total would be 6/21 or 0.29, still significantly higher than the KONUS 11/12 sample.

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Further confirmation of a significantly higher frequency of short bursts has come from the SIGNE experiments on Venera 11 and 12. Diyachkov et al.¹² report that a distinct class of bursts with durations less than 0.25 s comprises 25% of the total, 49 confirmed events. The SIGNE experiments utilized the same trigger integration time as the KONUS 13 and 14 instruments¹³, 0.25 s. If experiments were to utilize trigger integration times as short as the average FWHM of short bursts, ~ 0.1 s, the percentage of short bursts then detected may be even higher, perhaps $\sim 40\%$, affording a better opportunity to study these fast events.

The event duration distribution of Fig. 1 suggests that distinct physical processes or different source populations are responsible for the long and short bursts. However, the anomalous 5 March 1979 burst, which has been suggested as a prototype for the short bursts³, probably represents a third category of γ -ray burst sources, given the possible extragalactic origin of this event and its clearly differing characteristics^{3,9}.

We thank Dr E. P. Mazets for providing us with unpublished results from the Venera 13 and 14 KONUS experiments.

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Deep crustal structure of south-west Rockall Plateau

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Two distinct types of passive continental margin are known to occur¹⁻³: one characterized by tilted and rotated fault blocks and crustal thinning (for example, refs 4-6) has clearly been formed by extension; the second and possibly more widely occurring type is characterized by the absence of tilted fault blocks and by a ubiquitous thick sequence of oceanward-dipping reflectors now known to be of volcanic origin^{3,7,8}. An oceanic origin for this volcanic sequence has been attributed, from magnetic and seismic evidence⁹⁻¹¹, to some kind of 'sub-aerial seafloor spreading'. However, evidence for the nature of the deep structure that would support an oceanic origin has been lacking. We report here new seismic reflection data and an interpretation of the deep crustal structure of the dipping reflector sequence of the south-west Rockall Plateau. Gravity models of the margin suggest that the dipping reflectors are underlain by stretched continental crust.

Over the past decade, the use of multichannel seismic reflection profiles and deep sea drilling results have led to a greater understanding of the tectonic evolution of passive continental margins, aided also by the development of simple theoretical models of the rift process (for example, refs 4, 5, 12, 13). In essence, stretching of the lithosphere revealed as rifting (that is, extension) in the upper crust is followed by thermal subsidence as spreading begins and rifting ends. Evidence of crustal extension has been amply documented by the large tilted and rotated fault blocks seen on the north margin of Biscay^{4,14}.

However, the increasing availability of multichannel seismic reflection profiles across passive margins worldwide has demonstrated a contrasting and possibly more widespread structural style in which tilted blocks are absent and in which an oceanward-dipping sequence of reflectors occurs. This type of margin is commonly but not always developed across stabilized cratonic belts, for example the Weddell Sea, south-west Africa, Argen-

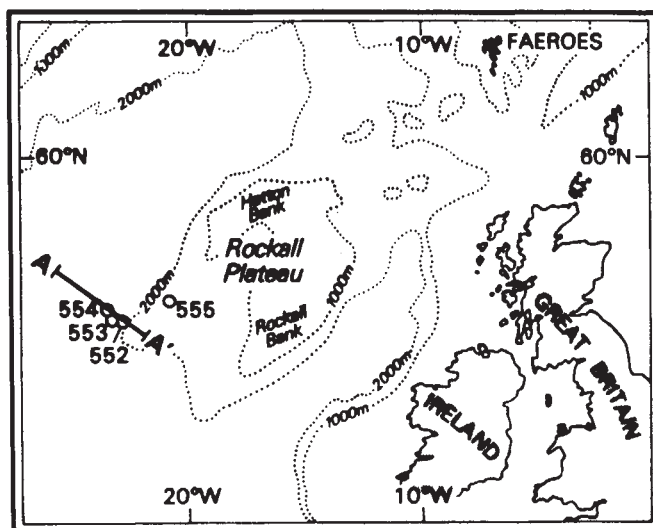


Fig. 1 Location of seismic/gravity profile across the south-west margin of Rockall Plateau: 552, etc. indicate location of IPOD Leg 81 sites. Leg 48 sites have been omitted for clarity.

tina and the east coast of the USA (refs 3, 15, 16 and D.G.R. et al., in preparation).

The south-west margin of the Rockall Plateau (Fig. 1) shows¹⁷ systematic variations in seismic character, reflector attitude and structure that allow simple subdivision into four zones (Fig. 2). Zone I comprises oceanic basement characterized by well defined and mapped sea floor spreading anomalies. The oceanic basement surface causes a strong, flat-lying reflector beneath which weak westerly and steeply dipping reflectors are evident. In the Zone I oceanic crust west of Rockall Plateau, these reflectors are diachronous¹⁸. The adjoining landward Zone II is a zone of elevated basement relief known as the 'outer high', partly overlapped by the oldest identifiable magnetic anomaly -24B¹⁸. The outer high is usually opaque on seismic profiles and generally is a zone of subdued relief, although relief of up to 500 m is present further along strike¹⁸. Clear dipping reflectors are not evident beneath the outer high. Eastward prograding clinoforms off the high indicate that it was a positive feature at

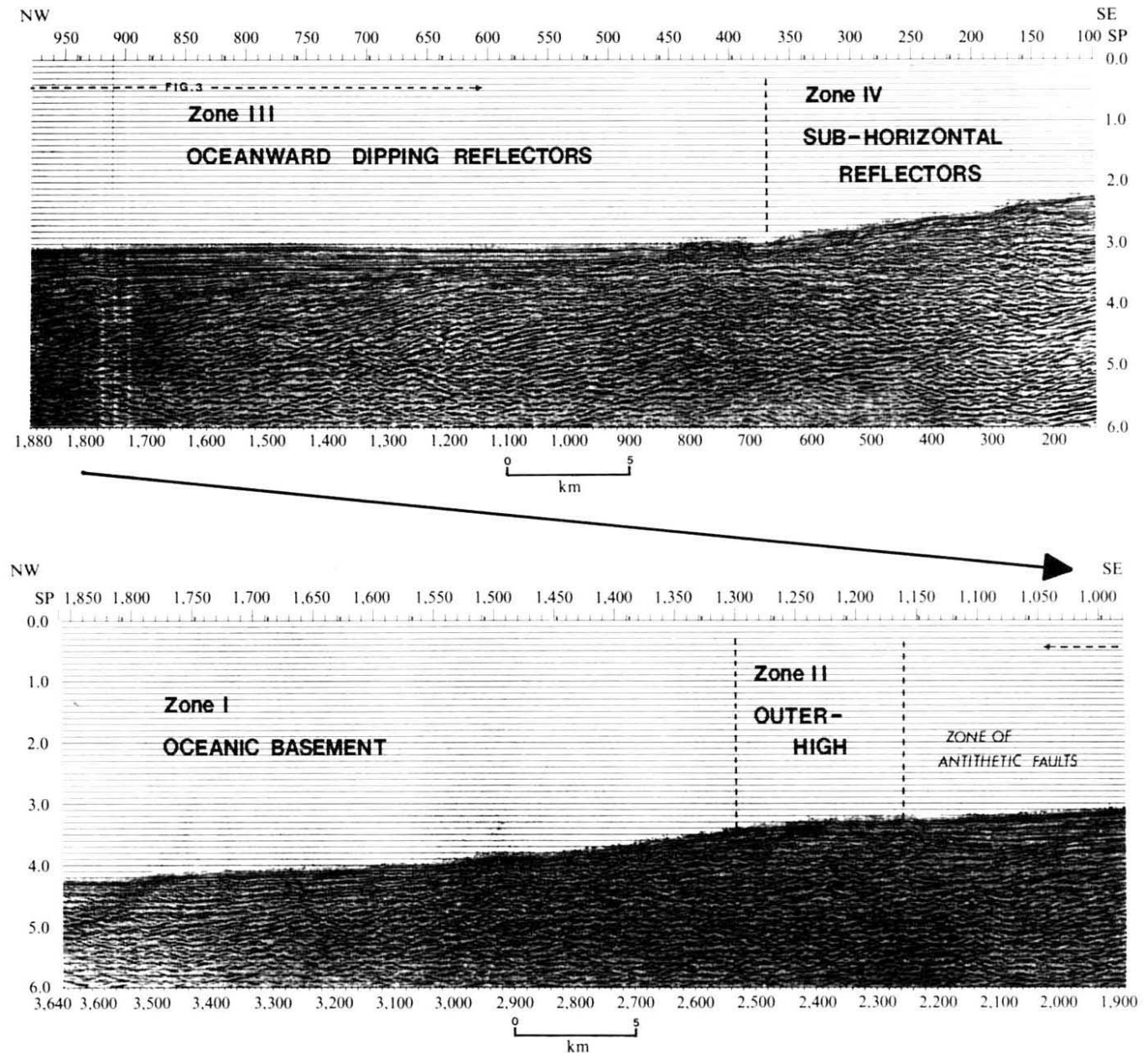


Fig. 2 Multichannel seismic profile across the south-west margin of Rockall Plateau. On other profiles¹⁸, eastward prograding clinoforms are observed on the east flank of the 'outer high'. Note the horizontal reflectors of Zone IV and the deep reflector extending as far as SP 600.

anomaly -24B time (ref. 18 and D.G.R. *et al.*, in preparation) (Fig. 2).

Between the edge of the outer high and the edge of Zone IV, a unique seismic pattern characterizes Zone III. The sub-horizontal reflectors of Zone IV flex downwards to form the characteristic sequence of oceanward-dipping reflectors. There is considerable variation in the attitude of the reflectors along strike: on some profiles the reflectors flatten westward but on others, westward divergence, possibly related to older topography or volcanic centres, is evident (D.G.R., in preparation). Typical seismic velocities in the dipping part of the sequence range from 3 km s^{-1} to 6 km s^{-1} in the lower part. As the outer high is approached, the dipping reflector sequence is upthrown to the west by a series of faults that dip eastward and are antithetic to the regional sense of extension. This structural configuration is exactly mirrored in the conjugate margin of south-east Greenland¹⁹. Deep sea drilling has established that the dipping reflectors are sub-aerial and submarine lavas⁷. The deep sea drilling results and the evolution of the margin will be discussed in detail elsewhere (D.G.R. *et al.*, in preparation).

The 'dipping reflector' type of margin shows several features that contrast dramatically with the Biscay margin. There is no evidence of tilted blocks downthrowing towards the ocean and the margin is dominated by a 5 km-thick pile of lava flows approximately 80 km wide that can be followed along strike for at least 500 km. Although the ubiquity of this type of margin has not previously been widely recognized, two mechanisms have been proposed. One, based on data from the Voring Plateau, considers that the volcanic pile formed by some kind of 'sub-aerial' seafloor spreading akin to that of Iceland^{9,10} and that lithosphere stretching is thus not involved. This model requires that the downdip ends of the lavas are joined to the feeder dykes of the mid-ocean crust. An alternative view considers that the volcanic pile was erupted onto crust instantaneously stretched in response to the thermal event responsible for voluminous lava production^{2,14}. A major problem in reconciling these hypotheses has been the lack of deep crustal structure data.

The new data, presented here, for the dipping reflector sequence of the south-west Rockall Plateau, consist of a multi-

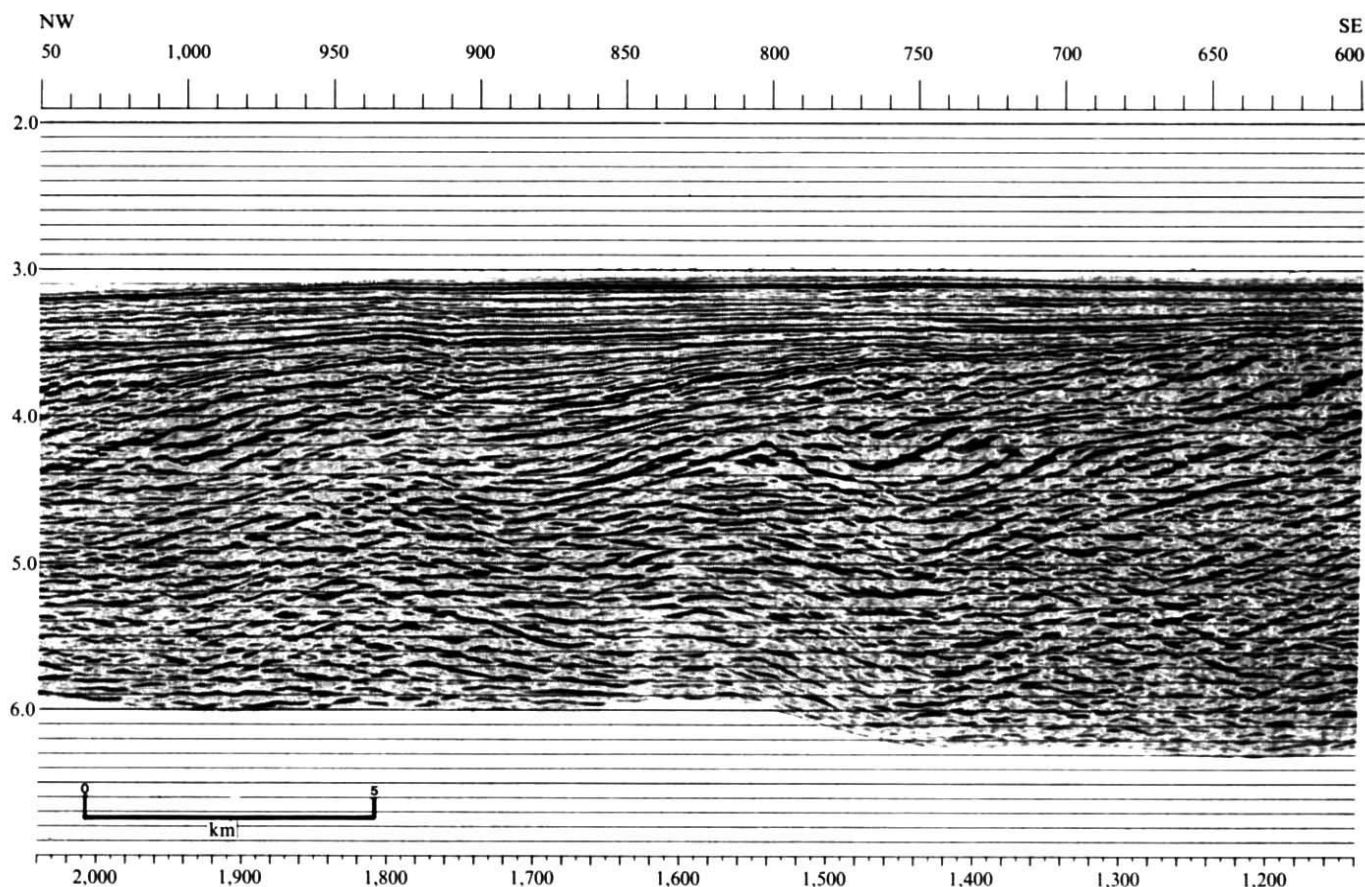


Fig. 3 Depth-migrated version of part of profile shown in Fig. 2. Note the antithetic faulting at SP 920. Depths in km.

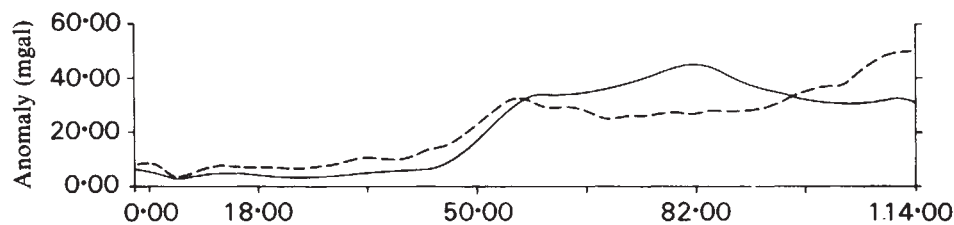
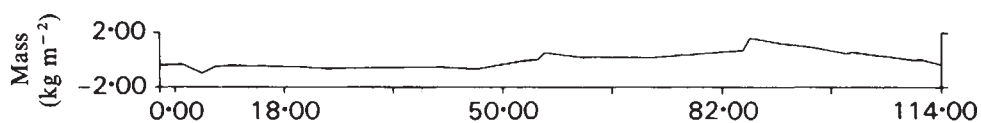
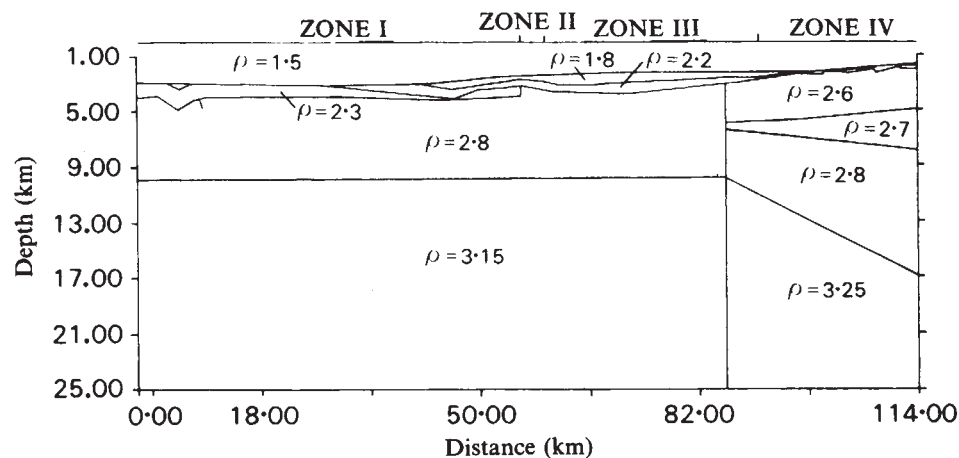


Fig. 4 Gravity model A of deep crustal structure along the line of the seismic profile. The model assumes oceanic crust to the Zone III/IV transition. Dashed line, observed gravity; solid line, computed gravity. Level of compensation, 35 km.



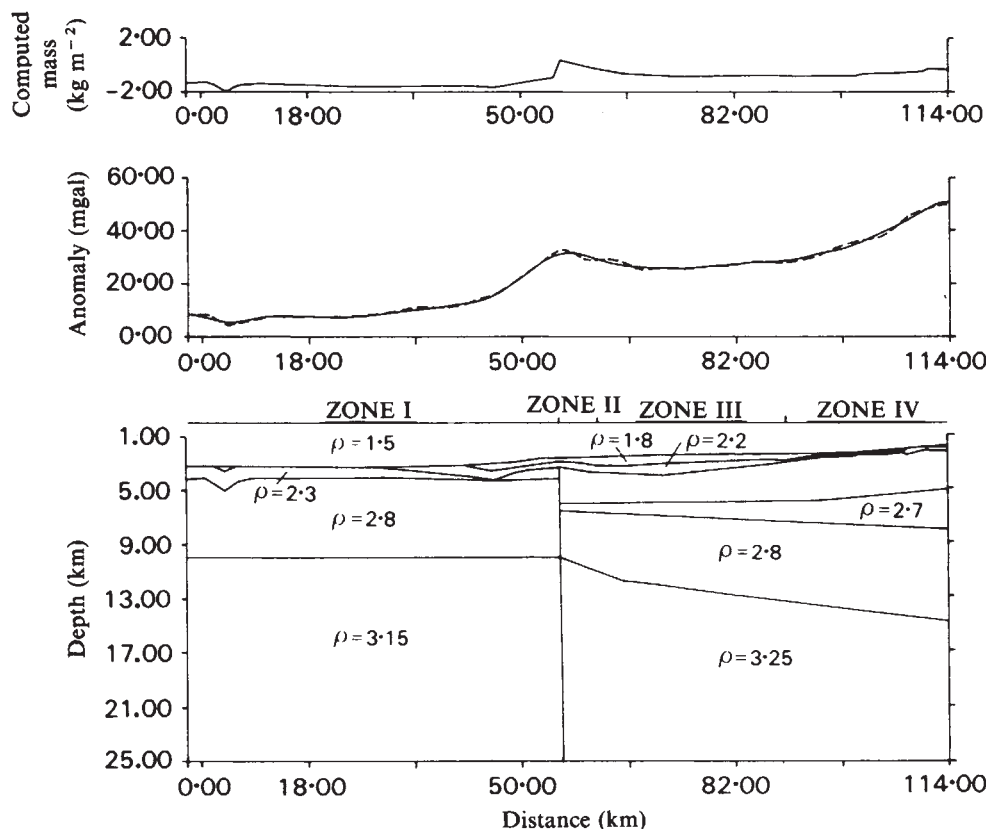


Fig. 5 Gravity model B of deep crustal structure along the line of the seismic profile. The model assumes that oceanic crust extends only to the inner edge of the outer high. Dashed line, observed gravity; solid line, computed anomaly. Level of compensation, 35 km.

channel seismic reflection (Fig. 2) and gravity profile across the margin. Unfortunately, processing failed to attenuate the strong multiple event from the top of the dipping reflector sequence. Nonetheless, a clear strong westerly dipping event can be followed beneath the dipping reflectors for 20 km. Using the gravity data obtained during the survey, we have simulated the gravity field using two models of crustal structure. These models respectively assume the presence of oceanic (model A) or continental (model B) crust beneath the lavas. The models are constrained by the depth-migrated deep reflection profile (Fig. 3) and short shallow refraction lines in the dipping reflectors and adjoining ocean crust (ref. 20 and D.G.R., unpublished).

Model A (Fig. 4) assumes a standard oceanic crustal section as far as the landward edge of Zone III, here assumed to be the continent-ocean transition. Landward thick lavas rest on thin continental crust. The difference between the observed and calculated anomaly is substantial and implies a lighter crustal column. We have attempted to construct a lighter crustal column using an Icelandic structure but find even greater disagreement.

Model B (Fig. 5) assumes that oceanic crust is only present as far east as the outer high and the landward limit of anomaly -24B. The landward edge of the outer high is here identified as the continent-ocean transition. The agreement between the observed and computed anomalies is good and indicates that the mass balance can be satisfied by the presence of stretched continental crust at depth.

It should be stressed that these models are crude in that deep refraction data are not available to constrain the position of Moho or velocity and density gradients in the lower part of the crust beneath the lavas. Nonetheless, the depth of penetration achieved in the seismic profiles places a considerable constraint on the possible range of variations in structure that can be realistically proposed in the lower crust.

Our results have considerable implications for the nature and position of the continent-ocean transition in such margins. In the conjugate margin of East Greenland, the oldest magnetic anomaly, -24B, overlaps the coastal basalts and dyke swarm²¹. Therefore, we have compared our model with the classical cross-sections across East Greenland²² and find good agreement. In Greenland, the accident of late Neogene uplift²³ has exposed

part of the section concealed beneath the deeply subsided basalts of Rockall (ref. 7 and D.G.R. *et al.*, in preparation). Continental crust is exposed beneath the basalts on the south shore of Scoresby Sund and within 20 km of the dyke swarm²¹. On the basis of our gravity model and by analogy with East Greenland, we argue, therefore, that the landward edge of the outer high represents the continent-ocean transition.

Although deep refraction data are needed to substantiate the nature of the continent-ocean transition, we tentatively offer a model for the evolution of this margin. Rapid and intense heating of thick, cold continental lithosphere was accompanied by pervasive dyke intrusion and voluminous eruption of lavas. Dyke intensity within the crust increased with time and towards the rift axis as the asthenosphere rose to break through the continental crust, forming the first oceanic crust represented by the outer high. In this model the dipping form of the reflectors reflects the response of the thermally weakened lithosphere to loading by lavas. The development of antithetic faulting adjacent to the outer high reflects thermal uplift immediately before the breakthrough of the asthenosphere and the onset of spreading.

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Dinoflagellate origin for sedimentary 4 α -methylsteroids and 5 α (H)-stanols

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Biological marker compounds provide useful tools for evaluating the depositional environments of Recent and ancient sediments and petroleum¹, largely through established relationships between the organic matter of sediments and source organisms^{1,2}. Such relationships are best tested and explored by investigating the lipid components of natural populations of autochthonous biota that can be clearly recognized as contributors to underlying bottom sediments. In this context we have studied the lipids of the freshwater dinoflagellate *Peridinium lomnickii* Woloszynska (order Peridiniales, class Dinophyceae) collected from the waters of Priest Pot³, a eutrophic lake in the English Lake District. Its distributions of both 4 α -methylsterols and 4 α -methylstanones closely resemble those of the underlying bottom sediments, demonstrating that dinoflagellates are important contributors of these sedimentary compounds. The 4-methylsteroidal hydrocarbons found in ancient sediments and petroleum^{4–6} are presumably diagenetic products of such 4 α -methylsteroids and therefore reflect dinoflagellate inputs to the original depositional environments. Furthermore, the prominence of 5 α (H)-cholestan-3 β -ol in *P. lomnickii* suggests that dinoflagellates may be the long sought, direct biological source of sedimentary 5 α (H)-stanols.

Dinoflagellates are major primary producers in the oceans^{7,8}, differing from other classes of both marine and lacustrine algae with respect to the dominance of 4 α -methylsterols among their sterol components and the uniqueness of certain of these 4 α -methylsterol structures. As the principal sterol of several marine dinoflagellates^{7,9,10} and other organisms with dinoflagellate symbionts¹¹, dinosterol (4 α ,23,24-trimethyl-5 α (H)-cholest-22-en-3 β -ol, Fig. 1, compound Ve) has been used as a biological marker for dinoflagellate contributions to sediments^{12–16}. Many other 4 α -methylsterols, including C₂₈ (Fig. 1, compound Va), C₂₉ (Vb, Vc), C₃₀ (Vie, Vf, Vd) and C₃₁ (Vg) components, variously occur in natural¹⁷ and cultured^{7,8,11,18–23} populations of marine dinoflagellates, including *Peridinium foliaceum*¹⁹. These compounds have been used as indicators of dinoflagellate inputs to marine sediments^{13,14}. The discrepancy between the prominence of such 4 α -methylsterols in subsurface sediments from the Namibian Shelf and the Peru upwelling area, and the lack of morphological remains of dinoflagellates (for example, cysts) in these sediments has led to speculation that these compounds may be derived from other phytoplankton, perhaps nanoplankton²⁴. There are few reports of the sterol composition of freshwater dinoflagellates²⁵ and 4 α -methylsterols have not been identified in freshwater algae. Hence, to understand better the contributions of these organisms to sediments, we have

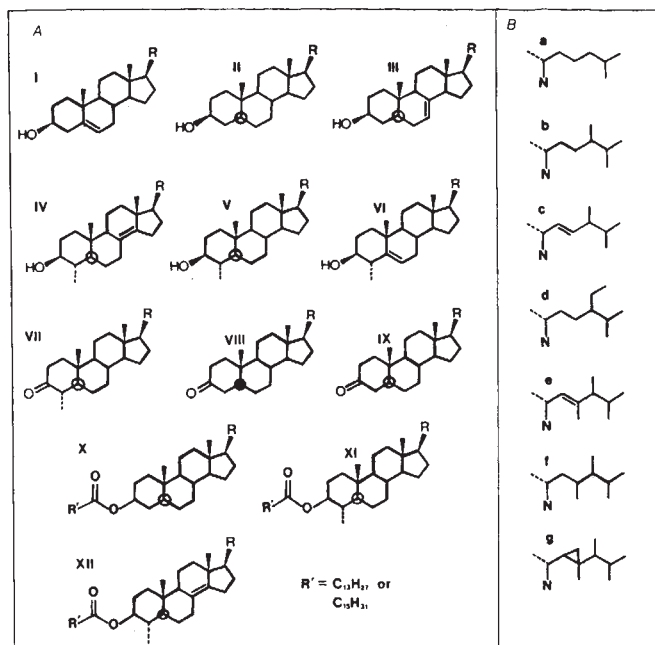


Fig. 1 Sterol, stanone and steryl ester structures. A, Steroid nuclei; R = steryl side chain C₁₅H₃₁ or C₁₇H₃₃ (a–g in B). B, Steryl side chain; N = steroid nuclei (I–XII in A).

examined and compared the lipid compositions of a natural population of the dinoflagellate *P. lomnickii*, and a short core of bottom sediment, collected from Priest Pot³.

P. lomnickii Woloszynska occurs, mainly during late winter months, in the plankton of small lakes in Poland, Holland, France and Central Europe²⁶. The organism is egg-shaped, with dimensions of 25–40 μ m $\times$ 22–35 μ m. A sample of *P. lomnickii* was collected from Priest Pot at a water depth of 1.2 m when present in high abundance (chlorophyll *a* > 1 mg l⁻¹). The concentrations of *P. lomnickii* in the epilimnion of Priest Pot and their high turnover rate suggest that substantial amounts of lipids derived from the dinoflagellates would have contributed to the underlying sediment. Preservation will also be aided by sedimentation into the anoxic hypolimnion² present during summer stratification of Priest Pot.

The lipids of *P. lomnickii* and those of the underlying sediment (0–6 cm) were extracted (CHCl₃/MeOH) by ultrasonication, followed by exhaustive extraction using a Soxhlet apparatus. Their total lipid extracts were separated by Al₂O₃ column chromatography and SiO₂ TLC. The various fractions obtained from the algae and sediment were analysed, after derivatization with bis(trimethylsilyl)trifluoroacetamide to yield trimethylsilyl (TMS) ethers for alcohols, by gas chromatography (GC) and computerized-GC-mass spectrometry (C-GC-MS), as previously described^{27,28}. Sterol and stanone identifications were based on GC retention times and individual mass spectra by comparison with previously published assignments^{13,14,27,28}.

Both the natural population of *P. lomnickii* and the bottom sediment contain similar suites of free sterols (Fig. 2), including desmethylsterols and 4 α -methylsterols. In addition, both contain the same range of 4 α -methylstanones (Fig. 3). A small amount of sterols was also present in an esterified form in *P. lomnickii*. Undoubtedly the most striking feature of these analyses is the high proportion of 4 α -methylsterols and 5 α (H)-stanols (Table 1) in both the organism and the bottom sediment. The fact that all of the 4 α -methylsterols recognized in *P. lomnickii* were present among the free lipids of the sediment in similar relative amounts (Fig. 2)²⁹, provides circumstantial evidence for the dinoflagellate origin of the sedimentary 4 α -methylsterols. One 4 α -methylstanol, 4 α -methyl-5 α (H)-cholest-8(14)-en-3 β -ol (Fig. 1, compound IVa), not previously reported in sediments, was detected in the sediments of Priest Pot (Fig. 2B), but not in *P. lomnickii*, although it has been identified in marine dinoflagellates⁸ and dinoflagellate

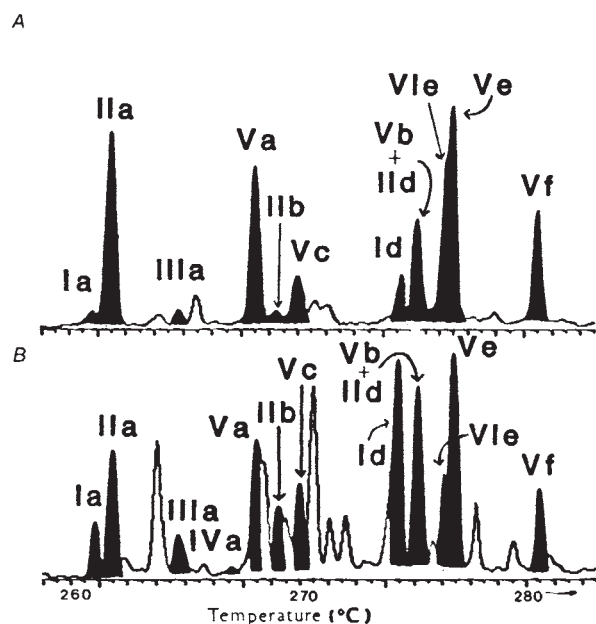


Fig. 2 Partial reconstituted ion chromatograms (RIC) from C-GC-MS analysis of the alcohols (as TMS ethers) of: *A*, *P. lomnickii* Woloszyńska; *B*, Priest Pot sediment (0–6 cm) free lipids. Sterol identities and structures are given in Table 1 and Fig. 1, respectively. GC conditions: 25 m OV-1 wall-coated Flexsil capillary column, programmed over 50–280 °C at 4 °C min⁻¹, after splitless injection at ambient temperature. MS conditions as described previously^{27,28} with spectra (*m/z* 50–550) recorded every second. The sterols present in *P. lomnickii* (designated peaks in *A*) were all recognized in the sediment (*B*), together with various other sterols (mainly C₂₇–C₂₉Δ⁵- and Δ⁷-sterols and 5α(*H*)-stanols) derived from other sources²⁹.

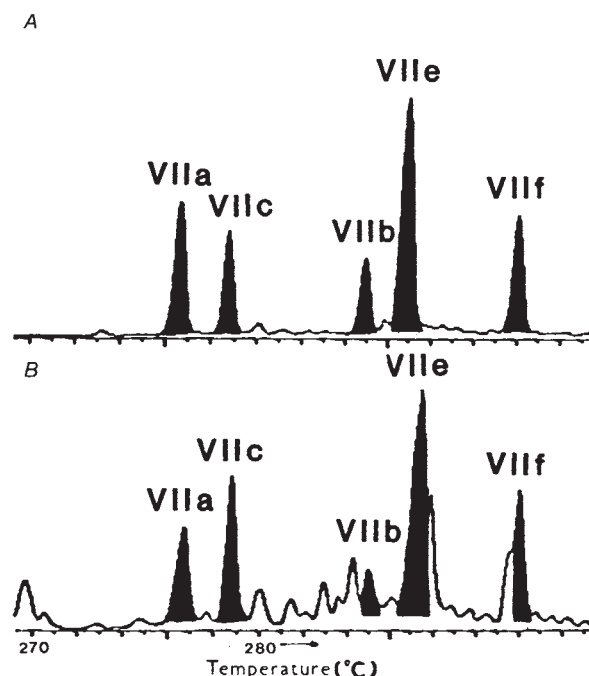


Fig. 3 Partial RIC from C-GC-MS analysis of the ketone fraction of: *A*, *P. lomnickii*; *B*, Priest Pot sediment (0–6 cm) free lipids. Stanone and structure identities are given in Table 1 and Fig. 1, respectively. GC and MS conditions as in Fig. 2. No Δ⁴-sten-3-ones were detected in *A* or *B* (see refs 14, 16, 24, 37). The 4α-methylstanones present in *P. lomnickii* (designated peaks in *A*) were all recognized in the sediment *B*, together with 5β(*H*)- and 5α(*H*)-stan-3-ones (VIII and IX in Fig. 1) derived from other sources or formed by *in situ* microbial oxidation of sterols^{29,36}.

zooanthellae¹⁶. It may occur in *P. lomnickii* at growth stages other than that sampled, or in another species of dinoflagellate inhabiting Priest Pot. Alternatively, this Δ⁸⁽¹⁴⁾-sterol may reflect methanotrophic bacterial activity in the sediment, since it has been recognized in *Methylococcus capsulatus*^{30,31}.

Previous reports have inferred bacterial *de novo* synthesis of 4α-methylsterols in lacustrine sediments^{32,33}, based on their occurrence in *M. capsulatus*^{30,31} and their absence both in poten-

tial external sources such as plankton³³ and in the products of sterol incubation in the sediments³³. The suggestion³² that bacteria like *M. capsulatus* are the source of dominantly ring-saturated 4α-methylsterols^{12–14,24} in marine sediments seems unlikely, as this methanotroph is reported to contain only nuclear unsaturated components^{30,31}. In addition, the only nuclear unsaturated 4α-methylsterol recognized in marine sediments is 4α-methyl-24-ethyl-5α(*H*)-cholest-8(14)-en-3β-ol

Table 1 Sterols and stanones present in a dinoflagellate population (*Peridinium lomnickii*) and in bottom sediments from Priest Pot (Figs 2, 3)

C _n identity	Structure (Fig. 1)	Quantitation <i>P. lomnickii</i>	Sediment
Sterols			
27 Cholest-5-en-3β-ol	Ia	1	7.1
27 5α(<i>H</i>)-cholestan-3β-ol	IIa	100	15.7
27 5α(<i>H</i>)-cholest-7-en-3β-ol	IIIa	6	1.5
28 4α-Methyl-5α(<i>H</i>)-cholest-8(14)-en-3β-ol	IVa	ND	0.4
28 4α-Methyl-5α(<i>H</i>)-cholestan-3β-ol	Va	86	25.3
28 24-Methyl-5α(<i>H</i>)-cholestan-3β-ol	IIb	3	0.5
29 4α,24-Dimethyl-5α(<i>H</i>)-cholest-22-en-3β-ol	Vc	15	14.4
29 24-Ethylcholest-5-en-3β-ol	Id	25	40.7
29 24-Ethyl-5α(<i>H</i>)-cholestan-3β-ol	IId }	45	31.5
29 4α,24-Dimethyl-5α(<i>H</i>)-cholestan-3β-ol	Vb }		
30 4α,23,24-Trimethylcholesta-5,22-dien-3β-ol	Vle	62 }	17.4 }
30 4α,23,24-Trimethyl-5α(<i>H</i>)-cholest-22-en-3β-ol	Ve	90 }	35.4 }
30 4α,23,24-Trimethyl-5α(<i>H</i>)-cholestan-3β-ol	Vf	64	17.4
Stanones			
28 4α-Methyl-5α(<i>H</i>)-cholestan-3-one	VIIa	70	1.8
29 4α,24-Dimethyl-5α(<i>H</i>)-cholest-22-en-3-one	VIIc	30	2.5
29 4α,24-Dimethyl-5α(<i>H</i>)-cholestan-3-one	VIIb	35	0.5
30 4α,23,24-Trimethyl-5α(<i>H</i>)-cholest-22-en-3-one	VIIe	100	5.9
30 4α,23,24-Trimethyl-5α(<i>H</i>)-cholestan-3-one	VIIIf	40	2.2

C_n, number of carbon atoms. Quantitation was determined by comparison of GC peak heights with those of standards: cholesterol (TMS) for sterols and *n*-C₂₈ alkane for ketones. *P. lomnickii* is expressed as % of most abundant component, and Sediment as μg per g dry sediment (0–6 cm). ND, not detected.

* Overlapping peaks; quantitation estimated from mass spectra.

(Fig. 1, Compound IVd)¹⁴, a known C₃₀ component of dinoflagellates⁸; by contrast, only C₂₈ 4 α -methyl $\Delta^{8(14)}$ -steroids were identified in *M. capsulatus*^{30,31}. Furthermore, the recognition of dinosterol in particulate traps^{34,35} demonstrates that sedimentary 4 α -methylsterols can originate from pelagic sources in the marine environment¹⁴, diminishing the need to invoke their *de novo* synthesis by bacteria^{32,33} in the underlying sediments.

Present evidence suggests that there are two major sources for the steroidal ketones found in sediments¹: formation by microbial degradation of sterols, as demonstrated by radiolabelling studies³⁶; and contribution as direct biolipid inputs from planktonic or other organisms, despite the scarcity of such reported occurrences³⁷. Dinosterone (4 α ,23,24-trimethyl-5 α (H)-cholest-22-en-3-one; Fig. 1, compound VIIe) has been identified in a cultured marine dinoflagellate²¹ and in marine sediments^{24,37,38} where, like dinosterol, it has been taken as an indicator of dinoflagellate inputs. The identification, in recent marine sediments^{24,37,38} and particulate matter traps^{34,35}, of other 4 α -methylstanones (for example, Fig. 1, compounds VIIa, VIIb, VIIc, VIIf), which were not known to occur in organisms, raised the possibility that these compounds were oxidation products of 4 α -methylstanols³⁷. Such 4 α -methylstanones and dinosterone are, however, major components of the free ketone fraction isolated from the sample of *P. lomnickii* collected in Priest Pot (Fig. 3A). Indeed, the distribution of 4 α -methylstanones in this freshwater dinoflagellate is very similar to that of the 4 α -methylsterols (compare designated peaks in Figs. 2A, 3A), reflecting the common biosynthetic origin of these groups of 4 α -methylsteroid components. The proposed role of dinoflagellates as contributors of 4 α -methylsteroids to sediments is supported by the occurrence, in the underlying sediments of Priest Pot (Fig. 3B), of all of the 4 α -methylstanones recognized in *P. lomnickii* (Fig. 3A), together with various 4-desmethylsterones derived from other sources²⁹.

We infer from these data that the freshwater dinoflagellate *P. lomnickii* is the major source of 4 α -methylsterols and 4 α -methylstanones in the lacustrine environment of Priest Pot. Other species of freshwater dinoflagellates may also contain these lipids, by analogy with their widespread distribution in marine dinoflagellates. Presumably, dinoflagellates can contribute 4 α -methylstanones as well as 4 α -methylstanols to underlying sediments in both marine and lacustrine environments. Dinoflagellate blooms may be aggregated both vertically and horizontally in a stratified lake³⁹ and dispersion of aggregates by wind-induced destabilization of the water column can occur readily. In Priest Pot the bloom of *P. lomnickii* occurred intermittently between early April and mid-June. The biolipid imprint of dinoflagellates would, therefore, be expected to be associated with brief pulses of organic matter deposited as thin layers of sediment. Hence, plankton sampling in lakes at monthly intervals, as made during the study of sterols in Lake Lemana³³, might miss a dinoflagellate bloom and its component 4 α -methylsterols. Alternatively, the plankton population of Lake Lemana may have changed in recent years, as 4 α -methylsterols were not detected in the upper 3 cm of the sediment³³.

The 4 α -methylsteroids, notably dinosterol (Fig. 1, compound Ve), preserved in certain ancient marine sediments, have been attributed to dinoflagellate inputs^{40,41}. The identification of 4 α -methylstanols and 4 α -methylstanones in *P. lomnickii* suggests that the occurrence of such compounds in ancient freshwater sediments, such as the lacustrine Messel shale⁴², may also reflect contributions from dinoflagellates. A complex, systematic sequence of diagenetic reactions modify sterols and sterones in sediments, generating a variety of steroidal hydrocarbons¹. 4-Methyl analogues of such compounds occur^{4,6}, presumably formed from 4-methylsterol and 4 α -methylsterone precursors by similar processes and additional isomerization at C-4^{1,4}. This relationship suggests that the widespread occurrence of 4-methylsteroidal hydrocarbons in ancient sediments and petroleum may reflect contributions from dinoflagellates to their original environments of deposition.

Various suggestions have been made to account for the higher relative abundance of 5 α (H)-stanols (Fig. 1, compound II) compared with Δ^5 -stenols (compound I) in sediments than in organisms¹; also, for the observed increase in stanol/stenol ratios with sediment depth^{43,44}. The present consensus of geochemical opinion suggests that preferential degradation of stenols relative to stanols can account for the increase with depth in the stanol/stenol ratio^{43,44}, and that in many environments this process is more significant than *in situ* microbial reduction of stenols to stanols⁴⁵. Also, it is now recognized¹ that 5 α (H)-stanols are major sterols of certain organisms. For example, 5 α (H)-cholestan-3 β -ol (Fig. 1, compound IIa) comprises 5.6% and 7.5% of the total sterols of the marine dinoflagellates *Noctiluca milialis*¹⁷ and *Pyrocystis lunula*⁷, respectively. In *P. lomnickii* collected from Priest Pot, the high abundance of 5 α (H)-cholestan-3 β -ol (Fig. 2A, compound IIa) is striking, as its dominance over cholest-5-en-3 β -ol (Fig. 2A, compound Ia). This feature is partly preserved in the underlying sediment (Fig. 2B), where contributions of cholest-5-en-3 β -ol from other sources appear to diminish the C₂₇ stanol/stenol ratio. Similarly, 24-ethyl-5 α (H)-cholestan-3 β -ol (compound IIId) is a significant component of both *P. lomnickii* and the sediment (Fig. 2, compound IId). These data suggest that the freshwater dinoflagellate *P. lomnickii*, and presumably other dinoflagellates, are important sources of sedimentary 5 α (H)-stanols. A direct source for 5 α (H)-stanols in several sediments can be inferred from the lack of correlation between the 5 α (H)-stanol/ Δ^5 -stenol ratios for different sterol moieties^{1,13,14,27,28}, a feature that excludes the uniform or systematic reduction of stenols to stanols. For marine sediments from the Black Sea^{12,13} and elsewhere^{14-16,24,46} which contain 4 α -methylsterols, it seems likely, therefore, that a portion of their 5 α (H)-stanols could be attributed to contributions from dinoflagellates.

The lipids of *P. lomnickii* also include series of steryl esters, namely stanyl (compound Xa) and 4 α -methylstanyl (compounds XIa, XIb, XIc, XIId) tetradecanoates (R' = C₁₃H₂₇ in Fig. 1) and hexadecanoates (R' = C₁₅H₃₁ in Fig. 1). Such 4 α -methylstanyl esters with various side chains (compounds XIb, XIc, XIId and XIe) have previously been identified in a cultured marine dinoflagellate, together with 4 α -methyl- $\Delta^{8(14)}$ -steryl esters (compounds XIIa, XIIb and XIIId)⁸. Hence, the prominence of esterified 4 α -methylsterols in Black Sea sediments¹³ is probably a further reflection of the importance of inputs from dinoflagellates to these sediments⁴⁷.

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1,8-Dimethylnaphthalene as an indicator of petroleum maturity

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The maturation of sedimentary organic matter along the evolutionary pathway which eventually gives rise to petroleum is of fundamental importance in petroleum geochemistry. The use of aromatic hydrocarbons as indicators of maturity of associated organic matter is now attracting increasing attention^{1–7}. We have examined the distributions of the 10 isomeric dimethylnaphthalenes (DMNs) in organic extracts of sediments and in a range of crude oils; the relative abundance of the 1,8-isomer (1,8-DMN) was found to change markedly and in a systematic manner both with depth of sediment burial (and temperature) and with values of other maturity parameters. We also report a correspondence between the 1,8-DMN ratio and two established molecular maturity parameters in crude oils selected to span a wide range of geographical location, geological age, and organic matter source type. The variation in the 1,8-DMN ratio is such that the maturation of samples ranging from immature oils to mature condensates can be determined.

We previously reported a protocol for quantitative analysis of all DMN isomers in oils and source rock extracts⁸, and we have now developed the procedure to be applicable to determination of the 1,8-DMN ratio on a routine basis. Briefly, an aromatic fraction is first separated from the source rock extract by liquid chromatography; an alkylnaphthalene fraction is then isolated by thin layer chromatography, and final analysis is effected by high-resolution capillary gas chromatography using a cross-linked OV-17 fused silica column.

Figure 1 shows plots of the logarithm of the 1,8-DMN ratio against sediment depth of the two sequences. In each sequence, the abundance of the 1,8-DMN relative to the other isomeric DMNs decreases in an orderly fashion with increasing depth. This change is attributed to the effects of increasing temperature with depth facilitating chemical reactions which result in preferential depletion of the least stable 1,8-DMN isomer⁹, relative to all the other more stable isomers. The fact that the relative

Table 1 Geochemical data for oil samples used in this study

Sample No.	Name	Location	Age of reservoir formation	Source C ₂₇ /C ₂₉ steranes
(1)	Barrow 6200 crude oil	W. Australia	Cretaceous	0.9
(2)	Barrow 6700 crude oil	W. Australia	Jurassic	0.8
(3)	Barrow condensate	W. Australia	Jurassic	0.5
(4)	Shangli crude oil	China	Cretaceous	2.3
(5)	Lambert crude oil	W. Australia	Triassic	0.8
(6)	Mangara crude oil	Chad	Cretaceous	—
(7)	Muderong crude oil	W. Australia	Cretaceous	0.8
(8)	North Apoi crude oil	Nigeria	Miocene	1.1
(9)	Vern crude oil	Denmark	Jurassic	1.5
(10)	Safaniya crude oil	Saudi Arabia	Cretaceous	1.2

abundance changes by almost two orders of magnitude in going from the shallowest (cooler) to the deepest (hotter) samples illustrates the great sensitivity of this isomer to its thermal environment.

Some particulars of the oils used in our study are presented in Table 1. Differences in geographical location and geological age of the reservoir formations are immediately apparent. The source type of each oil was inferred from the ratio of its C₂₇/C₂₉ sterane components: increased levels of C₂₇ steranes indicate increased contribution of marine organic matter¹⁰. Figure 2 shows plots of the logarithm of the 1,8-DMN ratio against two other maturity indicators based upon biological marker com-

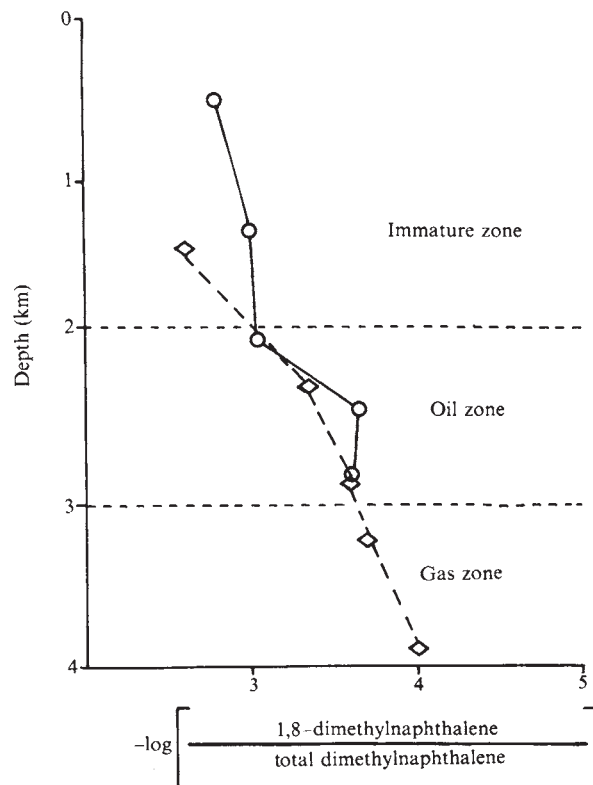


Fig. 1 A plot of the relative abundance of 1,8-dimethylnaphthalene against depth for two Jurassic sedimentary sequences from the Carnarvon Basin, Western Australia. Samples are from Cape Range No. 2 (○) and Barrow No. 1 (◇) wells. The oil zone was inferred from vitrinite reflectance ($R_0 = 0.6$ – 1.1) and from Rock-Eval pyrolysis data.

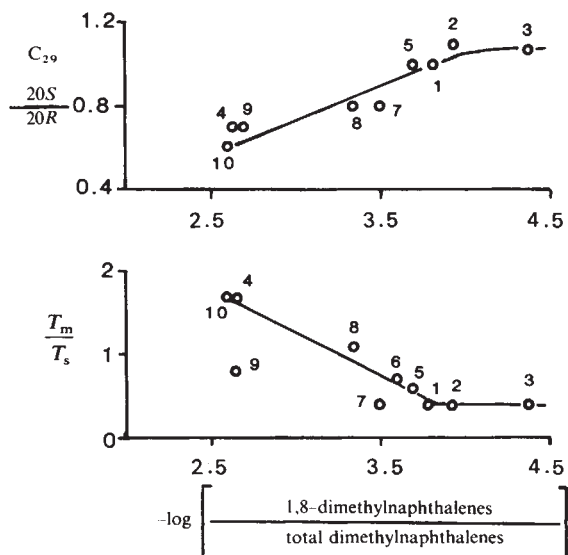


Fig. 2 Plots showing a comparison of thermal maturation parameters based on sterane ($C_{29}(20S/20R)$) and triterpane (T_m/T_s) biological marker compounds with the relative abundance of 1,8-dimethylnaphthalene for crude oils. Refer to Table 1 for identification of crude oil samples. Concentrations of steranes in sample 6 were insufficient for reliable measurements.

pounds which have been established recently. The first of these indicators is based on the observation¹⁰⁻¹⁴ that the concentration of C_{29} sterane with the $20R-5\alpha(H), 14\alpha(H), 17\alpha(H)$ -configuration decreases relative to that of the corresponding $20S$ isomer with increasing maturity. Thus the ratio $20S/20R$ increases with increasing maturity, reaching a maximum value of approximately 1.1 in the oil window. The second of these maturity parameters is based upon the change in relative concentrations of two C_{27} hopane isomers. The concentration of $17\alpha(H)-22,29,20$ -trisorhopane (T_m) decreases with respect to that of $18\alpha(H)-22,29,30$ -trisorhopane (T_s) with increasing levels of maturation; accordingly, the ratio T_m/T_s has been developed as a maturity parameter¹⁵ but is somewhat less reliable than the parameter based on steranes. Both plots shown in Fig. 2 reveal a good correlation for most samples within the range where the biological marker parameters are applicable. The location of the point for sample 3, a condensate, is interesting: evidently this sample has undergone maturation beyond the dynamic range of the biomarker maturity parameters, and this is reflected in the 1,8-DMN ratio. Thus the new 1,8-DMN parameter appears to have potential as a maturity indicator for high maturity zones where formation of condensates and gases occurs; the maturity of such zones may not be measured satisfactorily by the biological marker maturity parameter. Again, values of the 1,8-DMN ratio do not appear to be susceptible to change in source type of organic matter. A further factor in favour of the 1,8-DMN ratio as a maturity parameter is that DMNs are well represented even in light crude oils and condensates; they occur at greater concentrations than the biological markers of such samples, and hence are likely to be more representative of the geological history of the samples. Furthermore, they occur at concentrations convenient for analysis by gas chromatography: expensive gas chromatography-mass spectrometry facilities are therefore not required to measure this parameter.

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Accelerator radiocarbon dating of evidence for prehistoric horticulture in Illinois

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With the development of direct detection radiocarbon dating, which uses an accelerator as part of a highly selective mass spectrometer, it is now possible to determine the age of milligram samples of organic materials¹⁻⁵. One application of accelerator dating is in evaluating scanty, sometimes controversial evidence for early horticulture throughout the world. We have now used the technique to date small samples of carbonized, cultivated plant remains from archaeological sites in Illinois. The results, reported here, establish (1) that squash was introduced by 7,000 yr ago, 2,500 yr before eastern North American records previously reported; (2) that horticulture involving indigenous plants had begun by 4,000 BP in eastern North America with domestication of *Iva annua*, a small-seeded annual; (3) that anomalous discoveries of Archaic period maize represent contaminants; and (4) that introduction of maize by initial Middle Woodland times (~2,000 BP) is questionable.

Misidentification of plant materials, post-depositional disturbance, misinterpretation of archaeological context, and sample cross-contamination can all lead to spurious evidence of early horticulture⁶. Bulk extraction of plant remains from sediments, such as by flotation, has increased the quantities of plant remains that are routinely examined, thereby increasing the likelihood that a contaminating specimen will be found occasionally, though perhaps not recognized as such. Because critical specimens from bulk samples are usually first identified in the laboratory, the possibility for more than routine inspection of field context is lost. With the advent of accelerator dating, contextual uncertainties can now be effectively resolved by determining the ages of critical specimens directly. Previously, this was seldom possible because most specimens are too small to determine ¹⁴C content by conventional β -decay counting.

Nine samples of carbonized plant remains from five archaeological sites (Fig. 1) were selected for accelerator dating. Table 1 gives the results. Samples were selected for which the

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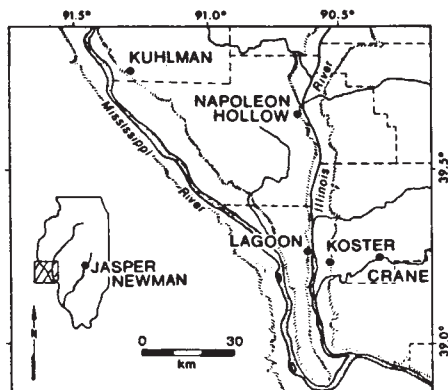


Fig. 1 Archaeological site locations in Illinois.

difference between the age of a specimen, based on apparent context, and its probable age, if present as contaminant, was great enough to be resolved even with a large standard error in the accelerator age determination.

Dating was conducted on the University of Rochester's tandem Van de Graaff accelerator by methods described in refs 1–5. Carbon isotope ratios were obtained by analysis of a carbon-containing ion beam produced in a caesium sputter source. Samples used in the source were homogeneous mixtures of pretreated and powdered archaeological charcoal and powdered copper. Abundances of ^{12}C and ^{13}C atoms were determined by Faraday cup current readings; ^{14}C atoms were counted individually in a gas-filled ionization counter. Background ^{14}C was shown to be negligible by measuring ^{14}C in a 40,000-yr-old charcoal sample.

Age determinations, corrected for natural and accelerator isotopic fractionation, were accomplished by comparing $^{12}\text{C}/^{13}\text{C}/^{14}\text{C}$ ratios in the archaeological specimens with those in an AD 1850 wood standard. Isotopes in each sample were measured cyclically several times and compared with the standard at least twice. The quoted uncertainties are 1σ error estimates, based on either the sample variance of repeated measurements or the statistical uncertainties resulting from the limited number of atoms counted, whichever was greater.

Records of Archaic period squash (*Cucurbita* sp.) from west-central Illinois consist of carbonized rind fragments from seven distinct occupations at four sites. Three samples were dated by direct carbon detection, retaining part as a voucher: from Koster and Napoleon Hollow sites (the oldest samples) and from Lagoon. Each date confirms the independently assessed age of the specimen's context (Table 1).

Koster⁷ and Napoleon Hollow⁸ were occupied repeatedly during the Holocene. Situated on colluvial fans, these sites have well-marked stratigraphies that are substantiated by numerous β -decay dates. The oldest squash, about 7,000 yr old, was recovered from Koster occupational horizon 8B, $3\frac{1}{2}$ –4 m beneath the surface. From 516 g of horizon 8B charcoal, seven squash rind fragments weighing 27 mg were identified in three of 239 flotation samples. The slightly more recently dated squash from Napoleon Hollow was 2.6 m below the surface within the older of two Middle Archaic occupational strata.

Accelerator dating substantiated the age of squash from a near-surface context at Lagoon⁹, where ring fragments were found with a *Sedalia* point in a shallow pit truncated by ploughing. *Sedalia* points are a diagnostic artefact type of the site's dominant occupation, a 4,000-yr-old Late Archaic Titterington component.

Other records of squash rind between 7,000 and 4,000 BP are supported by β -decay dates on associated charcoal. They include squash in a midden of Koster horizon 8A ($6,860 \pm 80$ BP, ISGS-835) and in three dated pit features: Napoleon Hollow's upper Middle Archaic occupation ($5,350 \pm 70$ BP, ISGS-938) and Titterington occupation ($4,060 \pm 75$ BP, ISGS-823) and Kuhlman site's Titterington component ($4,010 \pm 130$ BP, ISGS-982). (Investigations at Kuhlman were directed by H. Hassen,

Center for American Archeology¹⁰.) Elsewhere in eastern North America, the presence of pepo squash (*Cucurbita pepo*) after 4,400 BP is firmly established^{11–13}.

Epidermal lithocysts and a regular organization of large isodiametric cells in cross-section make small *Cucurbita* rind fragments easy to identify. No wild *Cucurbita* is native to Illinois, but *Cucurbita foetidissima* extends eastward into Missouri (perhaps even farther east during the Hypsithermal climate of 7,000 BP?), and it is adventive into Illinois along railroads¹⁴. The carbonized archaeological rinds are thicker on the average than rind of *C. foetidissima* but are comparable with *C. pepo* var. *ovifera*, a weedy ornamental variety probably similar to the ancestral form of *C. pepo*¹⁵. Thus, the Archaic squash is presumably *C. pepo*, the only cultivated squash documented in prehistory from the midwestern United States¹⁶.

Pepo squash was probably introduced from Mexico or south-east Texas¹⁷. It self-propagates readily and may seldom have required replanting. The prehistoric rind fragments are too small to observe effects of selection under cultivation. Presumably, early squash had thin flesh, perhaps unaltered through selection to eliminate bitterness¹⁸. The seeds would have been edible, but the plant's value as containers, floats and rattles may have exceeded its importance as food¹⁹.

Iva annua L. (marshelder), whose achenes contain an edible oily kernel, was one of the species cultivated before maize-based horticulture. By 5,700–4,900 BP at Koster (horizon 6, Helton phase), *Iva* was economically important, though not necessarily cultivated, as the achenes are wild-sized²⁰. From Napoleon Hollow there is evidence that domestication had begun by 4,000 BP. Among 79 *I. annua* achenes from a shallow pit, measurable specimens have a mean length of 4.2 mm ($n = 44$) and a mean width of 2.8 mm ($n = 58$)¹⁵ corrected by Yarnell's method²¹ for loss of pericarp and shrinkage from carbonization. Dimensions exceed mean achene size from modern wild stands ($\sim 2.8 \times 2.2$ mm)²⁰ or from older archaeological contexts¹⁵ and are comparable with those of domesticated *Iva* from Salts Cave, Kentucky, at 3,000 BP²². Current evidence suggests that achenes this large would not be produced as a phenotypic response to favourable growth conditions; nor would they result from selective harvesting or processing in an undomesticated population²⁰.

The *Iva*-containing pit was 0.9 m below the surface at the base of a buried Titterington Archaic cultural stratum. Dispersed charcoal from the pit was dated by the β -decay method, and one of the large achenes was selected for accelerator dating to test for intrusiveness from an overlying post-Archaic cultural stratum. The results confirm the Titterington Archaic association (Table 1).

Compelling evidence for cultivation by 4,000 BP exists for no other plant of eastern North America's native small-seed horticultural complex¹⁵. Notably, seeds of two members of this complex, *Chenopodium bushianum* Aellen (goosefoot) and *Helianthus* sp. (sunflower), occurred with *Iva* in the Napoleon Hollow pit. Possibly they were also cultivated at this early time, but the seeds could not be distinguished from wild types¹⁵.

From Koster, carbonized maize cupules, kernels and glumes were identified (1) in nine flotation samples from four cultural horizons, 9D to 9A, which are stratigraphically below the squash-yielding levels and have β -decay dates of 8,220–7,800 BP; and (2) in a sample from a Late Archaic stratum, above which a β -decay date of 2,980 BP was obtained⁷. Separation from potential sources of contamination seemed as good as one could hope for at an archaeological site, especially for the older, deeply buried strata. However, since an Archaic age for maize in eastern North America would have broken radically with current archaeobotanical syntheses, only dates on the maize itself could provide the needed strong test of age. Accelerator dating shows conclusively that it was a late prehistoric or recent contamination (Table 1).

The maize was a contaminant in 0.3% of the Archaic flotation samples. Cross-contamination in processing probably introduced it into the older samples. Post-depositional disturbance may account for it in the more shallowly buried Late Archaic

Table 1 Accelerator dates and associated β -decay dates

Specimen	Site, provenience	Specimen age, accelerator date (yr BP)*	Associated β -decay dates (yr BP)*	Expected age if contaminant† (yr BP)
Squash rind	Koster, Sq. 41-32 (hor. 8B)	7,100 $\pm$ 300 (NSRL-298)	6,910 $\pm$ 100 (ISGS-1082) 6,960 $\pm$ 80 (ISGS-848) 7,000 $\pm$ 80 (ISGS-809)	<1,350
Squash rind	Napoleon Hollow, Sq. 36-46	7,000 $\pm$ 250 (NSRL-299)	6,630 $\pm$ 100 (ISGS-786)‡ 6,730 $\pm$ 70 (ISGS-937) 6,800 $\pm$ 80 (ISGS-817)	<2,000
Squash rind	Lagoon, Feat. 102A	4,300 $\pm$ 600 (NSRL-303)	4,010 $\pm$ 150 (ISGS-798) 4,030 $\pm$ 75 (ISGS-804)	<2,000
<i>Iva annua</i> achene	Napoleon Hollow, Feat. 20	4,500 $\pm$ 500 (NSRL-297)	3,920 $\pm$ 90 (ISGS-933)‡ 4,060 $\pm$ 75 (ISGS-823)	<2,000
Maize	Koster, Sq. 193-61 (hor. 9B)	250 $\pm$ 300 (NSRL-295)	7,920 $\pm$ 150 (ISGS-923)	<1,350
Maize	Koster, Sq. 411-12 (hor. 4B)	600 $\pm$ 400 (NSRL-296)	2,980 $\pm$ 70 (ISGS-956)§	<1,350
Maize	Jasper Newman, Feat. 33 (= 13) 56-64 cm	450 $\pm$ 500 (NSRL-300)	2,030 $\pm$ 140 (ref. 37)‡ 2,000 $\pm$ 140 (ref. 37)	<900 (600-500)
Maize	Napoleon Hollow, Sq. 73-01	0 $\pm$ 300 (NSRL-301)	1,800 $\pm$ 70 (ISGS-904) 1,970 $\pm$ 70 (ISGS-935)	<1,350
Maize	Crane, 56E1-03, 56E2-03B	1,450 $\pm$ 350 (NSRL-302)	1,710 $\pm$ 70 (ISGS-1081) 1,750 $\pm$ 70 (ISGS-951) 1,870 $\pm$ 70 (ISGS-1107) 2,050 $\pm$ 70 (ISGS-898)	<1,200

* Based on ^{14}C half life of 5,568 yr, corrected for isotopic fractionation, uncalibrated for atmospheric ^{14}C fluctuations, 0 BP = AD 1950.

† Based on previously documented antiquity in region and on archaeological components represented at site.

‡ From same feature or flotation sample as accelerator-dated specimen.

§ From above maize in 2 $\times$ 2 m test square, horizon 4A.

context, which was 0.7 m beneath maize-bearing Late Woodland/Mississippian levels. Schoenwetter²³ also identified three maize pollen grains in samples from Archaic horizon 6. Their authenticity as Archaic pollen is no more credible than that of the carbonized maize before the accelerator age determinations.

It was once widely postulated that the cultural complexity of Hopewellian and contemporaneous Middle Woodland groups at about 2,000 BP required a productive dependable subsistence base attainable only through maize horticulture. Recent intensive flotation sampling has not substantiated this proposition^{15,24,25}. Also, increases in $^{13}\text{C}/^{12}\text{C}$ ratios in human bone due to maize consumption appear to post-date Middle Woodland times^{26,27}. Establishing whether maize was even present at 2,000 BP remains important for selecting among models of horticultural development. Was it a more recent introduction? If so, maize might have rapidly become a major crop because it was immediately judged to be a superior food resource. Or was there an earlier lengthy period of petty utilization? This alternative has led to suggestions that time was required to select strains of reliable and high yield in eastern North American habitats or that demographic pressures among Late Woodland populations were central in transforming economies from primary dependence on traditional, nutritionally adequate foods towards more productive but nutritionally unbalanced maize-based systems²⁸⁻³².

Several maize discoveries possibly dating to ~2,000 BP or even earlier are reported from eastern North America^{16,19,33-35}. For many of these, a potential for contamination is obvious, especially because Middle Woodland sites are often unstratified near-surface deposits reoccupied later by maize-using groups. A residuum of cases has been widely accepted¹⁶, but in each instance the quantity of maize is small and the maize itself is undated. Harlan and de Wet⁶ caution that, in evaluating the quality of evidence for origin and dispersal of cultivated plants, confidence in the authenticity of an association is reduced when the quantity of material is small, however secure the context may seem. We could not accept that the introduction of maize into eastern North America by 2,000 BP had been established incontestably. Therefore, we selected maize for accelerator dating from three of the best apparent Middle Woodland associations in Illinois: from Jasper Newman, Napoleon Hollow and Crane sites.

At Jasper Newman, Gardner excavated several cob fragments and detached cupules from lower levels of a pit truncated by ploughing^{33,36}. A β -decay date of 2,030 BP from the pit³⁷, the presence of 186 Havana sherds, and apparent protection of the pit fill from subsequent disturbance all supported a Middle Woodland association. Still, this was the only Middle Woodland context at the site in which maize was identified (L. W. Blake, personal communication). The Illinois State Museum provided us with cupules from the pit for dating. The date, 450 BP, is younger than 2,030 BP by a 3 σ level and is close to dates of 620-520 BP obtained from three Mississippian pit features at the site³⁷. Thus, accelerator dating has disposed of the case formerly regarded by at least one archaeobotanist³⁸ as 'the earliest convincingly dated maize in the East'. Can any other early maize record, then, be considered established beyond reasonable doubt?

At Napoleon Hollow, maize was discovered from a 25-m² trash dump in a surface A-horizon on a steep forested slope never disturbed by ploughing. Two carbonized maize cupules and a glume were present in one flotation sample. Dates of Middle Woodland age on dispersed charcoal (Table 1) correspond with the inferred age of artefacts from the deposit, which include sherds from 76 Middle Woodland vessels. In this unusual location for an archaeological site, evidence was lacking for later prehistoric or historic activities plausibly serving as a source of carbonized maize. Nevertheless, an accelerator date on one cupule plainly establishes that it was a recent contaminant.

At Crane, a Middle Woodland base settlement³⁹, 9% of midden flotation samples contained carbonized maize²⁰. However, remains of very minor Late Woodland occupations were also present in the essentially unstratified midden. (1) A Jersey Bluff occupation (post-1,200 BP) was represented only by midden debris and accounted for less than 4% of sherds in surface collections. Maize is common at other Jersey Bluff sites²⁰. (2) An ephemeral occupation at 1,400-1,300 BP lacked maize in the few associated pit features. Closely related White Hall/Weaver components in the region also lack maize⁴⁰.

The sample for accelerator dating—a kernel and cupule fragment—was the only maize possibly within pit fill. Even this association was uncertain because of A-horizon soil development which obscured the pit's upper limits. All of 207 sub-plough-zone pottery sherds within 2 m of the sample were

Middle Woodland types; overlying plough zone contained 127 Middle Woodland and 5 Jersey Bluff sherds. The date obtained on the maize, 1,450 BP, is younger than expected for the Middle Woodland component, for which a range of 2,050–1,700 BP would be appropriate. The 1 σ error does not, however, preclude a Middle Woodland origin.

Of 220 Middle Woodland pit feature samples and an equal number of midden samples, only 23 midden samples contained maize. A selective disposal pattern in which Middle Woodland maize was excluded from refuse pits seems improbable, as no other kind of carbonized material or artefact class exhibited such a pattern. Most plausibly, the maize in the midden was left by the Jersey Bluff inhabitants.

In conclusion, the present results suggest that squash, which was introduced by 7,000 BP, was the first cultivated plant of eastern North American Indians, probably as a minor element in a subsistence economy in which nuts were the focus of plant utilization²⁰. Cultivation of indigenous species can be traced back at least to 4,000 BP, since by that time a trend towards increasing size in *Iva annua* achenes had begun, reflecting a continuing process of the plant's domestication⁴¹. Major dependence on cultivated eastern North American plants and introduced cucurbits occurred by 3,000 BP in some areas^{42,43} and at about 2,050 BP in west-central Illinois (coincident there with the appearance of Hopewellian Middle Woodland societies)^{24,44}. We question whether maize was part of the 2,000 BP horticultural complex, and this position is supported by the negative results of accelerator dating. There is reasonably convincing evidence from pit fill contexts in Tennessee that maize had been introduced by 1,550 BP^{11,45}, and maize seems definitely to be present in west-central Illinois at 1,350 BP¹⁵. By 1,000 yr ago, maize was a major crop throughout much of eastern North America.

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Holocene age of the Yuha burial: direct radiocarbon determinations by accelerator mass spectrometry

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The view that human populations may not have arrived in the Western Hemisphere before about 12,000 radiocarbon yr BP^{1,2} has been challenged by claims of much greater antiquity for a small number of archaeological sites and human skeleton samples. One such site is the *Homo sapiens sapiens* cairn burial excavated in 1971 from the Yuha desert, Imperial County, California^{3–5}. Radiocarbon analysis of caliche coating one of the bones of the skeleton yielded a radiocarbon age of 21,500 $\pm$ 1,000 yr BP⁴, while radiocarbon and uranium series analyses of caliche coating a cairn boulder yielded ages of 22,125 $\pm$ 400 and 19,000 $\pm$ 3,000 yr BP, respectively⁵. The late Pleistocene age assignment to the Yuha burial has been challenged by comparing the cultural context of the burial with other cairn burials in the same region⁶, on the basis of the site's geomorphological context and from radiocarbon analyses of soil caliches.^{7,8} In rebuttal, arguments in defence of the original age assignment have been presented^{9,10} as well as an amino acid racemization analysis on the Yuha skeleton indicating an age of 23,600 $\pm$ 2,600 yr BP¹¹. The tandem accelerator mass spectrometer at the University of Arizona has now been used to measure the ratio of ¹⁴C/¹³C in several organic and inorganic fractions of post-cranial bone from the Yuha *H. sapiens sapiens* skeleton. Isotope ratios from six chemical fractions all yielded radiocarbon ages for the skeleton of less than 4,000 yr BP. These results indicate that the Yuha skeleton is of Holocene age, in agreement with the cultural context of the burial, and in disagreement with the previously assigned Pleistocene age of 19,000–23,000 yr.

Both sides in the Yuha controversy have recognized that resolution of the controversy would require radiocarbon analysis of the bone itself, but this was initially delayed because conventional ¹⁴C methods would have required destruction of much of the skeleton⁵. Subsequently, the theft of the major portion of the skeleton from the Imperial Valley College Museum in 1981¹² appeared to have precluded analysis of Yuha bone altogether. However, a few remnants of the skeleton still existed. (1) Thirty-four grams of hand, foot, rib and unclassified bone fragments were overlooked when the major portion of the skeleton was stolen. These fragments remained in the possession of the Imperial Valley College Museum, El Centro, California (IVCM). (2) Approximately 1.3 g of bone fragments, from a sample of Yuha bone obtained in 1979, were in the possession

Table 1 Radiocarbon ages (yr BP)

Fraction	Sources of bone fragments		
	I (IVCM)	II (UCR)	III (USGS)
Total acid-insoluble organics	3,850 ± 250		
Total acid-soluble organics	1,650 ± 250		2,820 ± 200
Total bone inorganics	2,400 ± 300	2,650 ± 200	2,700 ± 200
Calcium carbonate film	2,800 ± 250*		
on bone (0.3 mm thick, mass 200 mg)	2,300 ± 300†		
Caliche fragment (I)	28,700 ± 2,000		
Caliche fragment (II)	3,050 ± 250		

* CO₂ prepared by reaction with 95% H₃PO₄.† CO₂ prepared by combustion at ≥900 °C with CuO.

of Pauline Stedt of the Department of Anthropology, University of California, Riverside (UCR). (3) About 5 g of unclassified bone fragments obtained for amino acid racemization analysis¹¹, were in the possession of James L. Bischoff, United States Geological Survey, Menlo Park, California (USGS). Although there was insufficient material left for radiocarbon analysis by gas proportional counting, there appeared to be enough for direct radiocarbon analysis by tandem accelerator mass spectrometry (TAMS). All of the Imperial Valley College Museum fragments, and 0.8 and 3.0 g, respectively, of the fragments held by P. Stedt and J. L. Bischoff were provided for dating at the TAMS facility at the University of Arizona.

All specimens were pretreated at the University of Arizona. Four types of fractions were prepared, two organic and two inorganic. The organic fractions consisted of the acid-soluble and acid-insoluble fractions obtained by extracting powdered bone with 0.3 M HCl. All adhering caliche was removed from the surface before treatment with the dilute acid. One of the inorganic fractions was carbonate CO₂ obtained from the bone by H₃PO₄ treatment; the other inorganic sample consisted of a 0.3 mm thick layer of caliche which was chipped from the surface of the bone fragments.

Each of the prepared chemical fractions was converted to CO₂ and then reduced to elemental carbon. The carbon samples from each chemical fraction were combined with iron powder and melted to form Fe-C beads suitable for mounting in the accelerator¹³. Ratios of ¹⁴C/¹³C in the targets were measured in a manner similar to that described previously¹⁴.

Table 1 gives the results of the ¹⁴C/¹³C ratios of the various inorganic and organic fractions, converted to radiocarbon ages. The errors quoted are standard deviations. Sample II (UCR) was so small that a target could be made only from the total inorganic bone fraction. From sample III (USGS), targets were made from the total inorganic bone fraction and from total soluble organics. The table also gives results obtained on several pieces of caliche curated at IVCM with the bones.

Several comments can be made concerning the results presented in the table.

(1) The radiocarbon ages of the total inorganic bone fractions produced from each of the three samples are, within errors, equal. This result supports our expectation that all three samples are from the same skeleton.

(2) The ages of the total acid-soluble organic fractions from two samples disagree by more than 2 s.d. The reason for this disagreement is not known, but both ages are clearly Holocene.

(3) The total acid-insoluble organic fraction is about 1,000 yr older than the other samples. This is consistent with measurements which we have made on 11,000 yr old mammoth bones¹⁵. That is, in the case of the mammoth bones, the acid-insoluble organic fraction appears to be older by about 1,000 yr than the acid-soluble organic and the inorganic fractions, and is equal to the known radiocarbon age of the mammoth skeleton.

(4) The age of the very thin (0.3 mm) layer of calcium carbonate removed from the bone is approximately equal to that of the total inorganic samples.

(5) Finally, as the Pleistocene age previously assigned to the Yuha skeleton was based at least partly on radiocarbon measurements made on caliche associated with the burial, we have

measured radiocarbon ages of several caliche samples associated with the burial site. Results of these measurements range from 2,300 yr BP to nearly 29,000 yr BP. From this we conclude that caliche samples found on or near the skeleton have various ¹⁴C components, and the inference of the age of the skeleton based on results obtained from associated caliche could lead to incorrect results. This observation is consistent with the conclusions of previous workers¹⁶.

Thus, direct measurements have been made of the ¹⁴C/¹³C ratios in six different targets made from three different chemical fractions extracted from three sets of bone fragments which were represented as having come from the Yuha skeleton. All of the measurements on these fractions yielded radiocarbon ages of the bones of less than 4,000 yr. We believe that the most reliable radiocarbon age measured was obtained from the total acid-insoluble organic fraction¹⁵, and is 3,850 ± 250 yr BP.

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First record of marsupials (Metatheria: Polyprotodonta) from the Oligocene in Africa

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Metatherian (marsupial) and eutherian (placental) mammals are end members of a complex, possibly common North American¹⁻⁴ or, less likely, a South American⁵ stock that diverged before the early late Cretaceous and underwent separate adaptive radiations establishing them as the two dominant mammalian groups (subclass Theria). By the later Cretaceous, marsupials were well situated in North America³ and were also present in South America⁶. Their oldest occurrences in Antarctica (later Eocene⁷) and Australia (Oligocene⁸) are artefacts of exceptionally poor fossil records of mammals on those continents and it is clear that the Metatheria must have been quite diverse in both areas (physically connected in pre-Eocene times⁹) very much earlier^{5,7}. Marsupials probably did not reach Europe much before their earliest record there in the early

Eocene¹⁰. The Metatheria have not been identified from Asia, nor until now from Africa. We now report the discovery of the first fossil remains of a didelphid marsupial in Oligocene rocks in North Africa.

Fossils of the first known African marsupials were found at fossil vertebrate quarry M¹¹, in the upper part of the Jebel Qatrani Formation¹², Fayum Depression of Egypt (Fig. 1). These are principally alluvial rocks deposited on a broad coastal plain^{13,14} that formed part of the southern margin of the Tethys Sea in Oligocene times (dated at about 32 Myr BP, T.M.B., E.L.S., J. G. Fleagle and J. D. Obradovich, unpublished data). Fossil crocodiles, turtles, rodents, browsing artiodactyls, hyracoids and quadrupedal arboreal higher primates were found associated with the marsupial remains. The Oligocene palaeoenvironment of the Fayum area was wet-tropical to subtropical and was characterized by seasonal rainfall that supported a diverse flora¹⁴.

Marsupial remains from Egypt consist of three lower jaw fragments: (1) a right mandible, Cairo Geological Museum (CGM) 40236 (Fig. 2), with the canine and first premolar roots, alveoli of the fourth molar, and crowns of the second premolar to the third molar; (2) a left mandible, CGM 40237, with molars 2 and 3 and part of molar 4; and (3) a left mandible, Duke Primate Center (DPC) 3120, with alveoli for molars 3 and 4 and a medially inflected angular process. In CGM 40236, the roots for the canine and first premolar are followed by two simple premolariform teeth and three fully molariform teeth. These are succeeded posteriorly by two alveoli for an eighth tooth. Those alveoli show that the eighth and last tooth was slightly larger than the seventh and almost certainly molariform. In the mandibular fragment CGM 40236, the last three molars are preserved; the anterior two of these are identical to the second and third molars in CGM 40236 and the last (most posterior) molar is slightly larger than any of the other molars and is also fully molariform. The tooth placements and morphologies in CGM 40237 show that the last tooth in that jaw is homologous with the last tooth (that preserved only by alveoli) in CGM 40236. Therefore, the animal whose mandibular dentition is represented by CGM 40236 and 40237 possessed a canine, three simple premolariform teeth, and four fully molariform teeth in the lower jaw (the most complete specimen, CGM 40236, is broken anterior to the canine root and the number of the incisors cannot be determined). DPC 3120 is a toothless left mandibular fragment with alveoli for the last two teeth and preserving part of a medially inflected angular process. This specimen was found at the same locality as CGM 40236 and 40237, it is identical in size and of comparable morphology with those specimens, and the size and spacing of alveoli in DPC 3120 indicate that the alveoli housed teeth of approximately the same relative sizes as the third and fourth molars in CGM 40237.

Lower jaws having three premolars and four molars in conjunction with a medially inflected angular process are a hallmark of generalized marsupials¹⁵ and are unknown in all eutherian mammals. Moreover, the relatively unspecialized tribosphenic lower molars with large trigonids and small talonids are identical to the general morphology of lower molars in members of the most generalized marsupial family, the Didelphidae, and clearly indicate placement of the Fayum marsupial in that family. Within the Didelphidae, the dental and mandibular morphologies of the three Egyptian specimens cannot be distinguished from those of the European genus *Peratherium* in the combination of: (1) the medially inflected angular process of the mandible^{16,17}; (2) the posterior increase in molar length¹⁸; (3) the posteriorly projecting molar hypoconulids, situated close to molar entoconids¹⁹; and (4) presence of a protoconid rising above the level of the metaconid¹⁰.

In the Old World, Tertiary didelphid marsupials otherwise occur only in Europe, where their temporal range is early Eocene^{20,21} to middle Miocene¹⁰. The ranges of French *Peratherium cuvieri* and *Peratherium elegans*, most like the Egyptian form, are late Eocene and early Oligocene, respectively¹⁰. Close

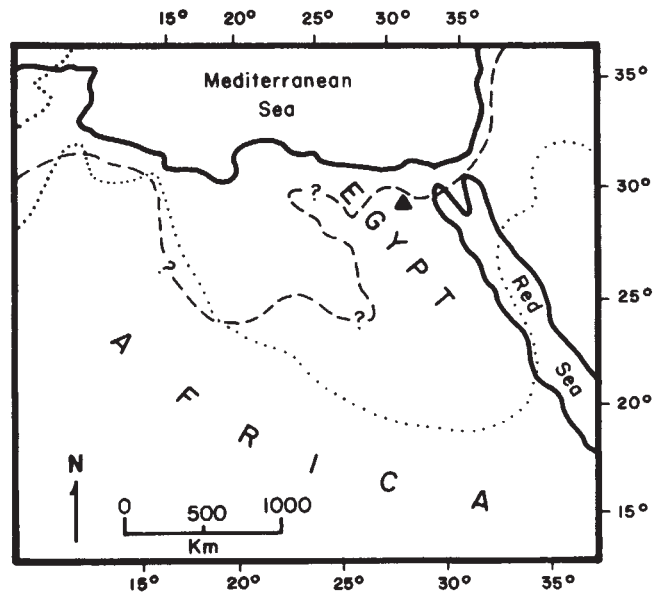


Fig. 1 Map of north-east Africa showing Fayum Depression (triangle), and Eocene (dotted line) and Oligocene (dashed line) marine strand lines.

similarity in form to these two European species indicates that entry of marsupials into Africa was ultimately from Europe probably no later than the early Oligocene and not much earlier than the late Eocene.

Palaeogeographical constraints support scanty palaeontological evidence that marsupials probably did not enter Egypt until at least the late Eocene, when the Tethyan regression was well underway, and they almost certainly arrived there somewhat later because the earliest terrestrial rocks in Egypt are early Oligocene. Palaeogeographical reconstructions of the Mediterranean Middle East for the late Eocene and early Oligocene do not support a direct land connection of Africa with Europe at that time^{22,23}, nor do they favour a less direct overland route from Europe via Asia across Paratethys and Tethys until at least

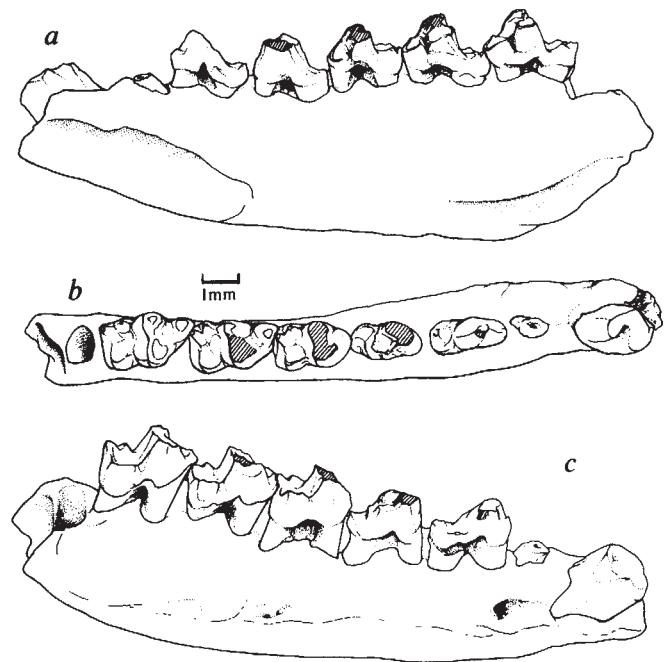


Fig. 2 Right mandibular fragment of marsupial from Oligocene rocks of Egypt; Cairo Geological Museum 40236, preserving second and third premolars, first to third molars, roots of canine and first premolar, and alveoli for fourth molar. a, Lingual; b, occlusal; and c, labial views.

the middle-late Oligocene, when much of the Middle East was an emergent area of erosion²⁴. A middle-late Oligocene date postdates the age of the Egyptian marsupials (unpublished data) and we therefore think it most likely that marsupials dispersed into Africa from Europe via the sweepstakes route (across the Tethys Sea) in the early Oligocene.

Direct land access to north-west Africa from Egypt was not possible until at least middle Miocene times, though a land route to East Africa might have been available during the Oligocene. The fact that marsupials are absent in both areas²⁵⁻³¹ suggests that they were unable to disperse there before they became extinct in Africa.

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A field study of the fallibility of polygraphic lie detection

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With the increasing use of polygraphic lie detection in the United States and United Kingdom it has become a matter of urgency to assess the accuracy and reliability of the technique. The main purpose of the present report, therefore, is to extend our earlier mock theft lie detection study¹ from the laboratory to the field. Again, as in the previous study, our findings show high false-positive and false-negative rates among polygraph interpreters. These data, with their unacceptably high error

rates, agree with the conclusions reached in a recent US Congress Office of Technology Assessment report² which reviewed the scientific validity of the polygraph. Hence, we conclude that the validity and reliability of polygraphic interrogation has yet to be established.

The subjects of the present research were 50 confessed thieves and 50 innocents who were interrogated at a leading polygraph company for the same thefts. The latter were suspected thieves who were subsequently cleared as a result of the confessions of the actual thieves, and they will be considered to have been verified 'truthful' during the polygraph questioning. The thieves will be considered to have been verified 'untruthful'.

The polygraph data of each of the 100 truthful and untruthful suspects were then randomly assigned to six polygraph interpreters who work at the same company as that at which the questioning occurred. In addition, the polygraphs of 20 unverified cases were also randomly assigned to the judges. These were not included in the data analysis, but merely served as filler or buffer cases to render the task more comparable to actual field conditions where it is unusual for polygraphers to be faced with interpreting clear-cut confirmed theft and no-theft protocols.

The information from each of the 120 polygraph examinations was presented to the interpreters. They were provided with polygraph chart data, which include all available physiological measures. The interpreters were to base their judgements on the protocol information dealing with the differences in physiological responses to the relevant ('Did you steal X amount?')

Table 1 Accuracy of polygraph judgements for six interpreters and the discriminant function analysis

	Valid positive	Valid negative	False positive	False negative	Overall accuracy
Interpreter					
1	78	60	40	22	69
2	64	62	38	36	63
3	70	82	18	30	76
4	82	66	34	18	74
5	80	50	50	20	65
6	82	56	44	18	69
Average interpreter	76	63	37	24	69
Discriminant function	74	72	28	26	73

Results are in per cent. The positive direction denotes a judgement of untruthful and the negative direction of truthful. Thus, a valid positive is a correctly identified theft subject.

and control ('Have you ever stolen anything?') questions having to do with the suspects' involvement (or noninvolvement) in the theft.

The polygraph data were then converted from analog to digital form. Next, using the digitized scores, a Hotelling T^2 test was used to determine whether the physiological data contain the necessary information to differentiate between truthful and untruthful responses. The results of this analysis were significant beyond the 0.01 level ($T^2 = 38.98$, d.f. = 10, 98), indeed indicating that the data contain the necessary discriminating evidence. An additional analysis involving a series of 95% simultaneous confidence intervals was conducted to ascertain whether any one particular polygraph cue contributed to this differentiation. The results suggested that the difference between the truthful and untruthful groups was due to the combined effect of the polygraph measurements.

We then performed a discriminant function analysis to discover the optimal weights for each of the physiological indices or cues in the polygraph protocol. The outcome of this analysis (Table 1) disclosed that on the average, optimal valid-positive, valid-negative, false-positive, false-negative and overall accuracy per cent, respectively, was 74, 72, 28, 26 and 73. Compared with these optimal discriminant functions, which capitalize on

Table 2 Inter-judge reliability correlation coefficients for physiological chart and for chart plus interview data

Physiological chart intercorrelations					
Interpreter	1	2	3	4	5
2	0.44				
3	0.49	0.56			
4	0.55	0.43	0.44		
5	0.48	0.24	0.41	0.35	
6	0.44	0.28	0.37	0.46	0.47

Chart and interview intercorrelations		
Interpreter	1	2
2	0.50	
3	0.51	0.42

chance errors and hence probably somewhat overstate the accuracy with which the two groups can be differentiated, we note in Table 1 that all but two of the six interpreters achieved valid-positive rates that outperformed the discriminant function, but only one interpreter outperformed the statistical analysis in the valid-negative category. This means that the interpreters are more likely to label a suspect untruthful than truthful, a bias that is particularly evident in the false-positive results which show that the misclassification rate of innocents is as high as 50%. The false-negative and overall accuracy rates of the interpreters are about comparable with the discriminant functions.

The false-positive rates shown in Table 1, although already high, became even more pronounced when three of the six interpreters were subsequently instructed to base their decisions on the physiological measurements plus interview information obtained during the interrogation. The average false-positive misclassification rate was then elevated from 37 to 39%, with one interpreter erroneously judging as many as 62% of the innocent suspects as guilty (these data are not shown in Table 1).

Inter-judge reliability coefficients for the physiological chart interpretations were then computed for the six judges (see Table 2). The average correlation was 0.43, with a range of 0.24–0.56. This average, when computed for the three interpreters who were later given access to the interview notes, improved somewhat to an average correlation coefficient of 0.48, with a range of 0.42–0.51. In both instances, however, the inter-judge reliabilities are modest at best and suggest that each of the interpreters uses the physiological and interview cues somewhat differently from his colleagues. This is surprising since they were all trainees at the same polygraph company.

To conclude, we note that professional polygraphers can differentiate between truthful and untruthful protocols, but can do so only with a moderate degree of success; that statistical analyses in the form of T^2 and discriminant function show that some of the information necessary for making these discriminations is contained in the polygraph data but that judges do not use this information optimally; and that the inter-judge reliabilities are low. The interpreters' modest success in false-negative and overall accuracy rates is reduced when the high false-positive rates are taken into consideration. This, together with the finding of low inter-judge agreement in the meaning of polygraph test cues, suggests that polygraphic lie detection as currently used is flawed and might best be replaced by an alternative method, perhaps the guilty knowledge test which numerous researchers have shown to be a more valid technique for uncovering deception^{3–5}.

Direct hyperpolarizing action of baclofen on hippocampal pyramidal cells

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Baclofen has been used as an antispastic agent for over a decade¹, yet its mechanism of action is still not fully understood. While early iontophoretic studies revealed a depression of neuronal activity^{2–4}, more recent studies have emphasized a presynaptic depression of transmitter release, both in the peripheral^{5,6} and central nervous system^{7–14}, possibly resulting from a blockade of calcium channels¹⁵. Although baclofen is structurally similar to the inhibitory neurotransmitter, γ -aminobutyric acid (GABA), none of its actions seem to be antagonized by the GABA antagonist, bicuculline. However, recent experiments have indicated that baclofen binds to a class of bicuculline-resistant GABA receptors, termed GABA_B receptors¹⁶. Here, we have analysed the action of baclofen on the membrane potential of CA1 hippocampal pyramidal cells *in vitro* and report that it directly hyperpolarizes these cells in a potent, stereoselective manner which is resistant to bicuculline methiodide. This response is associated with a decrease in neuronal input resistance and may involve an increase in potassium conductance.

Rat hippocampal slices were prepared and superfused as described previously¹⁷. The normal superfusion medium consisted of (in mM): NaCl 116.4, KCl 5.4, CaCl₂ 2.5, MgSO₄ 1.3, NaHCO₃ 26.2, NaH₂PO₄ 1.0, glucose 11. Either 2 M potassium methylsulphate (KMeSO₄) or 3 M potassium chloride was used to fill the recording microelectrodes, which had resistances of 60–120 M Ω . The membrane potential of the pyramidal cell could be shifted by passing current through the recording electrode using a bridge circuit. Drugs were applied either by superfusion or by iontophoresis. Iontophoretic pipettes were filled with either GABA (1 M, pH 5; Sigma) or ($\pm$)baclofen (25–100 mM, pH 3; Ciba-Giegy).

Application of baclofen either by superfusion or by iontophoresis had a very striking, stereotyped depressant action on CA1 hippocampal pyramidal cells; this action was elicited by iontophoretic baclofen onto the soma or the apical or basal dendrites, and was observed in over 80 pyramidal cells. Every cell to which baclofen was applied, responded with a hyperpolarization. In Fig. 1a, iontophoresis of GABA and baclofen into the pyramidal cell layer hyperpolarized the membrane potential and prevented the firing of a spontaneously active neurone. With a large iontophoretic current, the hyperpolarization to baclofen achieved was as large as 10 mV in amplitude. The action of baclofen was, as previously reported^{2–4}, much slower than the response to GABA: while GABA responses peaked in seconds, the baclofen responses always required tens of seconds to reach maximum amplitude, and to decay. The slow time course of action of baclofen could be due to the fact that, unlike GABA, it is not actively taken up¹⁶, consequently it may diffuse further. Indeed, the responses to identical iontophoretic current pulses of baclofen were considerably larger when elicited in the dendrites compared to the responses elicited by somatic applications. This suggests that baclofen may act primarily in the dendrites.

It is possible that the baclofen hyperpolarization resulted from a depression of tonic release of excitatory transmitter onto CA1 neurones (see, for example, refs 18, 19). However, tetrodotoxin (TTX), which blocks voltage-sensitive sodium channels, failed to reduce the GABA or baclofen response (Fig. 1a). The

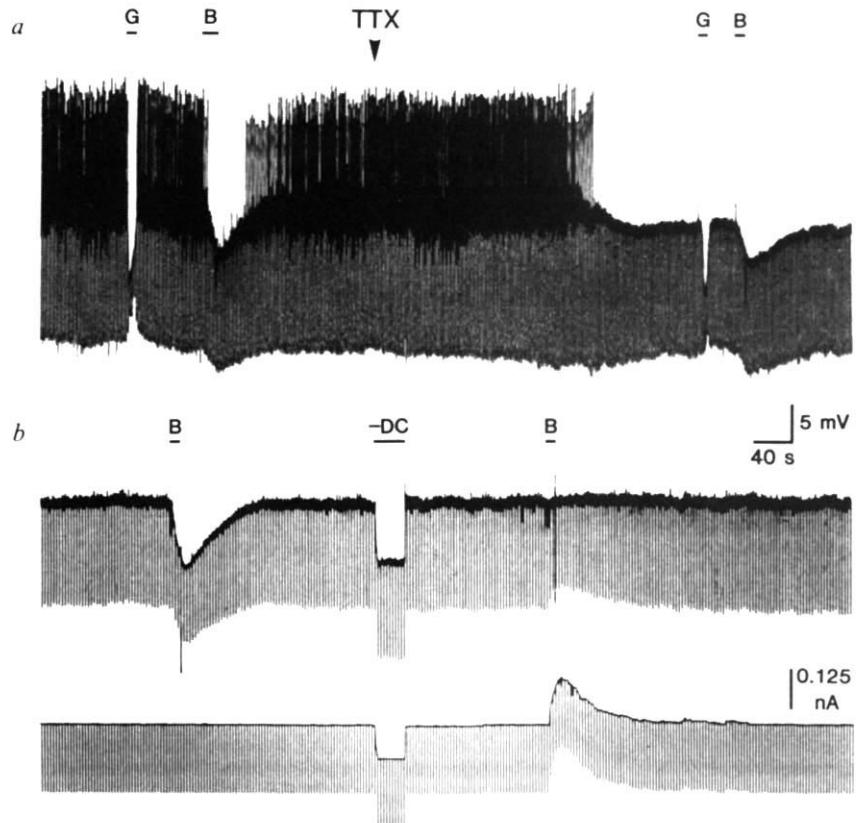
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Fig. 1 Baclofen directly hyperpolarizes hippocampal pyramidal cells. *a*, Iontophoresis of GABA (G) or ($\pm$)baclofen (B) induced a hyperpolarization and prevented the spontaneous action potentials of this neurone. These responses persisted after the superfusion of 0.5 μ M tetrodotoxin (TTX), which began at the arrowhead. Constant-current hyperpolarizing pulses were applied throughout. GABA was iontophoresed into the pyramidal cell layer with a 30 nA ejecting current. Baclofen was iontophoresed, less than 50 μ m away (in the apical dendrites), using a 60 nA ejecting current. Drugs were iontophoresed for the periods indicated by the bars. Resting membrane potential, -53 mV. *b*, Baclofen reduces the input resistance of hippocampal pyramidal cells. The input resistance is indicated by the size of the voltage deflections (top trace) produced by the constant-current hyperpolarizing current pulses (bottom trace). The membrane was also hyperpolarized with direct current (-DC) to control for inward rectification. The second baclofen response was clamped manually by injecting current (see bottom trace). ($\pm$)Baclofen was iontophoresed into the pyramidal cell layer with a 200 nA ejecting current. Resting membrane potential, -58 mV. No TTX was present in this experiment. The voltage and time calibration bars apply to *a* and *b*. Recording electrodes were filled with KMeSO₄ in both experiments. It should be noted that, in contrast to baclofen, to obtain a pure hyperpolarizing response to GABA, the iontophoretic electrode must be positioned very close to the soma of the recorded cell.



resistance of the baclofen response to TTX was observed in 16 cells. In addition, at a time when synaptic transmission was abolished by cadmium (100 μ M), an antagonist of voltage-sensitive calcium channels, the baclofen response was unaltered ($n = 3$ cells). Thus, we conclude that the hyperpolarizing action of baclofen resulted from a direct action on pyramidal cells.

We next determined whether the baclofen response is associated with a change in the neuronal input resistance by measuring the size of the voltage deflection elicited by constant-current hyperpolarizing pulses. Figures 1*a*, *b* and 2*b* show that an apparent decrease in input resistance occurred during the baclofen response, although it was always less pronounced than the decrease seen with GABA. This change in resistance was not due to inward rectification of the membrane because hyperpolarizing the membrane with steady current applied through the recording electrode did not change the input resistance to the same extent (see -DC in Fig. 1*b*). Furthermore, when the hyperpolarization was manually voltage-clamped (see second application in Fig. 1*b*), a decrease in input resistance was still evident. For hyperpolarizations of 5–7 mV the average decrease in input resistance was $15 \pm 5\%$ ($\pm$ s.d.) ($n = 15$ cells); this evidence indicates that baclofen increases the ionic conductance of the membrane and provides additional evidence that baclofen acts directly on the pyramidal cell membrane. An increase in the neuronal input resistance would be expected if the hyperpolarization resulted from a depression of tonic excitatory transmitter release.

We further investigated the response to baclofen with regard to previous reports of its stereoselective, bicuculline-resistant action in other systems. Hyperpolarizing responses were elicited by superfusing the (-) isomer at concentrations as low as 0.3 μ M; the (+) isomer, however, was much less potent. Figure 2*a* shows that, at a concentration of 10 μ M, the (+) isomer was less effective than the (-) isomer in eliciting a hyperpolarization. A stereoselective action has also been observed for both the postsynaptic⁴ and presynaptic^{11,12} actions of baclofen. In other experiments we found that the baclofen response was unaffected by doses of bicuculline methiodide (100 μ M), which strongly antagonized somatic GABA responses ($n = 8$) (Fig. 2*b*). These

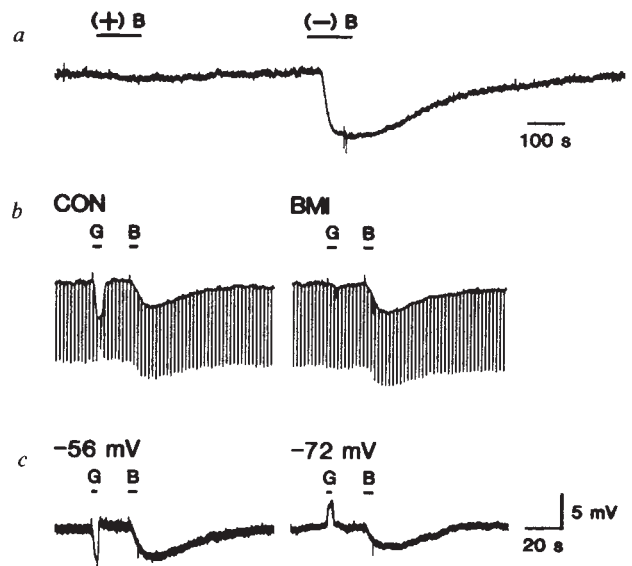


Fig. 2 Stereoselectivity of the baclofen response and its different sensitivity to bicuculline and membrane potential compared with GABA. *a*, Superfusion of 10 μ M of each of the optical isomers of baclofen reveals a marked difference in potency. Resting membrane potential, -61 mV. *b*, Selective depression of the response to iontophoretically applied GABA following a 10-min superfusion of 100 μ M bicuculline methiodide (BMI) (compare with control, CON). The GABA and baclofen iontophoresis electrodes were positioned less than 50 μ m from each other in the pyramidal cell layer. The GABA was applied by turning off the retaining current and the ($\pm$)baclofen was ejected with a 70 nA current. Constant-current hyperpolarizing pulses (0.2 nA) were applied throughout. Resting membrane potential, -63 mV. *c*, GABA and baclofen have different reversal potentials. GABA and ($\pm$)baclofen were iontophoresed into the pyramidal cell layer with 6 and 90 nA, respectively. The membrane potential was hyperpolarized from its resting level by direct current, and the applications repeated. The voltage calibration applies to all traces, and the time calibration in *c* applies to *b* also. Recording electrodes were filled with KCl in *a* and KMeSO₄ in *b* and *c*. Tetrodotoxin (0.5 μ M) was present in all of these experiments.

pharmacological results suggest that baclofen interacts with a receptor on pyramidal cells that is distinct from the classical bicuculline-sensitive GABA receptor.

Although it seems clear that baclofen directly hyperpolarizes pyramidal cells by an increase in the conductance of the membrane, the ionic mechanism underlying this response has not been fully elucidated. However, preliminary observations suggest that potassium ions are involved. When the recording electrode was filled with the potassium salt of the impermeant anion methylsulphate, the reversal potentials of the somatic GABA and baclofen hyperpolarizations were considerably different (Fig. 2c). The somatic GABA response reversed at -69.2 ± 3.4 mV ($n = 19$), while the baclofen response had an extrapolated reversal potential of -85.1 ± 2.6 mV ($n = 8$). It is widely accepted that GABA-mediated hyperpolarizations of pyramidal cell soma are primarily due to an increase in chloride conductance²⁰⁻²³. When the recording electrode was filled with potassium chloride the reversal potential for chloride at the soma became markedly less negative due to leakage of chloride ions from the electrode—as a result, somatic GABA responses became strongly depolarizing. In these conditions, however, the baclofen responses (see Fig. 2a) remained hyperpolarizing. The reversal potential of the baclofen response was not significantly different from that of the slow after-hyperpolarization that follows a series of action potentials, that is, -85.3 ± 2.7 mV ($n = 7$) (see ref. 24), which is considered to be due to a calcium-activated potassium conductance²⁴⁻²⁶.

The present results demonstrate that baclofen has a potent, direct, stereoselective, hyperpolarizing action on CA1 hippocampal pyramidal cells which is resistant to the GABA antagonist bicuculline methiodide. These pharmacological properties are similar to those resulting from activating GABA_B sites in other tissues⁵. In hippocampal pyramidal cells the baclofen response seems to involve an increase in conductance to potassium ions.

At present, a physiological role for this baclofen response is unknown. However, a synaptically evoked late hyperpolarizing potential, which follows a fast, GABA- and chloride-mediated inhibitory postsynaptic potential (i.p.s.p.) in hippocampal cells²⁷⁻³⁰, is now thought to be a bicuculline-resistant dendritic slow i.p.s.p.³¹. This slow i.p.s.p. has properties remarkably similar to the baclofen response described here. It has recently been reported that GABA can hyperpolarize pyramidal cell dendrites by a chloride-independent mechanism³². We are now investigating the possibility that GABA, acting on GABA_B receptors, may be the transmitter mediating the slow i.p.s.p.

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Prevention of natural motoneurone cell death by dibutyryl cyclic GMP

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Natural neuronal cell death is a well-described developmental phenomenon common to many nerve centres in a variety of animal species¹⁻⁸. Neuronal survival has been shown to depend on the presence⁹⁻¹¹ and size of the available target tissue^{12,13} and it has been suggested that neuronal survival is dependent on successful competition for either a limited number of synaptic sites^{1,14} or a limited amount of trophic factor(s)^{9,15}. In the lateral motor column of the lumbar spinal cord in the chick embryo, the period of axon elongation and innervation of the periphery has been shown to precede that of natural motoneurone cell death^{16,17}. While muscle contractile activity appears to regulate the extent of motoneurone death¹⁸, to date the intracellular molecular events that initiate and regulate the developmental process of natural neuronal cell death or, more importantly, neuronal survival are unknown. Our earlier studies suggested that either contact or association between spinal cord processes and muscle cells during neuromuscular junction formation *in vivo* leads to an increase in cyclic GMP in whole spinal cord¹⁹. We now show that treatment of chick embryos with the membrane-permeable cyclic GMP analogue, dibutyryl cyclic GMP during the period of natural motoneurone cell death prevents >58% of natural motoneurone cell death in the lumbar lateral motor column.

Drugs dissolved in Tyrode solution were administered to chick embryos (Harco reds × Partmer reds) by dropping 0.2 ml of solution onto the vascularized chorioallantoic membrane daily on embryonic days 5-9. Embryos were killed on day 10, staged²⁰ and weighed. The lumbar spinal cord was dissected, fixed in Carnoy's solution and stained *en bloc* with thionin²¹. The tissue was embedded in paraffin, serially sectioned at 8 μ m (6-day) or 10 μ m (10-day), and for every 10th section all large, dark-staining cells of the lateral motor column (LMC) containing at least one nucleolus were counted at $\times 400$.

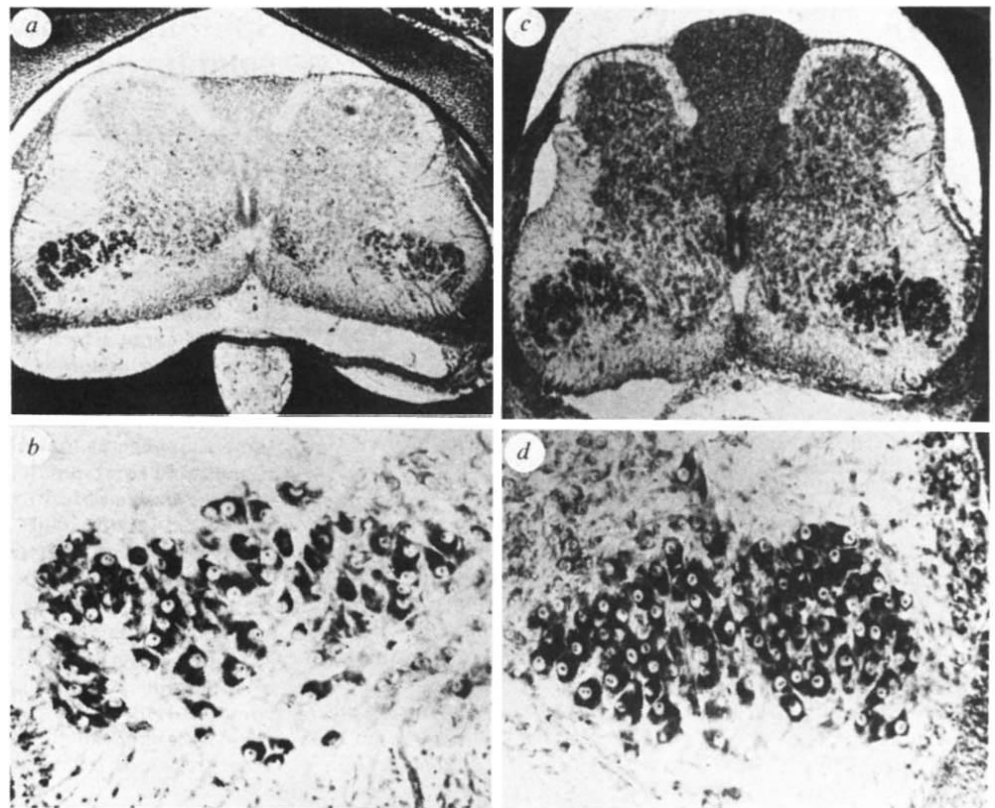
During normal development a peak of 20-24,000 motoneurons per LMC is observed on embryonic day 6 (refs 3, 18, 22 and our unpublished data). This number declines ~40% by day 10 to 13,000-14,000 cells per LMC. The embryos used in the experiments described here displayed a peak of $22,838 \pm 731$ (mean $\pm$ s.e., $n = 6$) motoneurons per LMC on day 6. The number of cells declined by 34% to $15,018 \pm 537$ ($n = 12$) on day 10. Daily injections of 0.91 mg of dibutyryl cyclic GMP per embryo from embryonic days 5 to 9 resulted in the survival of $19,540 \pm 516$ (mean $\pm$ s.e., $n = 12$) motoneurons per LMC on day 10, a loss of only 14% (Fig. 1).

The correcting of cell counts would normally not be necessary with the protocol used here because the section thickness is four to five times larger than the entire nucleolar diameter²³. However, because treatment with dibutyryl cyclic GMP significantly alters the percentage of cells that contain two nucleoli (see below), the effect of correcting the raw cell counts was determined. Separate correction factors for cells having one or two nucleoli were calculated from measurements of the mean

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Fig. 1 Cross-sections of spinal cords at the same segmental level from a 10-day control embryo (*a*, $\times 42$), enlargement of control LMC (*b*, $\times 130$), dibutyl cyclic GMP-treated embryo (*c*, $\times 42$), and enlargement of dibutyl cyclic GMP LMC (*d*, $\times 130$).



nucleolar diameter and the mean centre-to-centre inter-nucleolar distance for both control and treated cells^{23,24}. No significant difference between control and treated cells was observed for either parameter. The separate correction factors were applied to the percentage of cells in the population having one or two nucleoli, respectively. Corrected total LMC cells on days 6 and 10 were, respectively, 17,517 and 11,415, in close agreement with the corrected values reported by Hamburger³. The corrected cell count for dibutyl cyclic GMP-treated embryos was 15,251. These values represent a loss of 35% and 13% for control and treated embryos, respectively, which are not significantly different from the percentage loss values calculated from the raw data. Application of the correction factors did not alter the statistical significance of the difference between controls and treated animals ($P < 0.001$). The percentage of natural motoneurone death prevented by dibutyl cyclic GMP treatment was increased to 63%, which is within our observed counting error.

Equimolar injections of 8-bromo-cyclic GMP over the same time period were equally effective and resulted in $18,892 \pm 802$ (uncorrected) surviving motoneurons per LMC. The specificity of the response to dibutyl cyclic GMP was assessed by injecting equimolar amounts of sodium butyrate, guanosine, guanosine 5'-monophosphate and dibutyl cyclic AMP over the same period and performing cell counts as before on day 10. The total uncorrected number of surviving cells counted for each of the above groups was in the range of the 10-day controls, being, respectively, $16,516 \pm 1,048$, $16,053 \pm 1,043$, $15,631 \pm 1,042$ and $14,663 \pm 696$. The distribution of surviving neurones along the LMC appeared unaltered by dibutyl cyclic GMP treatment compared with controls (Fig. 2). Body weight also was not significantly altered by treatment, being 2.414 ± 0.107 g (mean $\pm$ s.e., $n = 5$) in controls and 2.578 ± 0.077 g ($n = 8$) in treated embryos ($P > 0.1$). Pittman and Oppenheim^{18,22} have also observed an increase in motoneurone survival, but as a consequence of neuromuscular blockade. The effect of dibutyl cyclic GMP on motility was therefore determined after the method of Pittman and Oppenheim¹⁸. Although this strain of chick embryos appears more motile than White Leghorns, no

significant difference in mean motility was observed (27.0 ± 1.7 ($n = 14$) and 25.4 ± 1.5 ($n = 9$) movements per min for control and treated embryos, respectively; $P > 0.5$).

These data demonstrate that $>58\%$ of natural motoneurone cell death in the LMC of the chick spinal cord can be prevented by exposure of the embryo to dibutyl cyclic GMP. In many other systems exogenous addition of this compound stimulates cellular processes known to be dependent on increased cellular concentrations of cyclic GMP²⁵. The specificity of the response was supported by the failure of other structurally related compounds to elicit a response. Specifically, the ineffectiveness of dibutyl cyclic AMP in this system was consistent with previous findings¹⁹ that no significant change in neuronal cyclic AMP levels was detectable over the period when cyclic GMP was observed to increase in spinal cord neurones. These findings collectively support the hypothesis that in normal conditions increased cellular cyclic GMP levels, either singly or in concert with other cellular functions, subserve motoneurone survival.

Close examination of the enlarged micrographs of the LMCs (Fig. 1*b, d*) of control and treated embryos shows a greater number of treated cells containing two nucleoli. Analysis of the sections of both LMCs of control and treated embryos yielded values (uncorrected) for the percentage of motoneurons containing two or more nucleoli of $28.6 \pm 1.1\%$ ($n = 5$, mean $\pm$ s.e.) and $36.7 \pm 1.8\%$ ($n = 7$), respectively ($P < 0.005$). These are minimum estimates, as some cells with two nucleoli would appear to have only one nucleolus if their nucleoli were split by sectioning. The application of correction factors to these data would only increase the difference between them as the factors would be proportional to the percentage of cells with two nucleoli. Since the confidence levels of this difference would also increase, we did not attempt to estimate the appropriate correction factors.

Nucleoli are sites of synthesis and processing of ribosomal RNA and are normally present in cells with high rates of protein synthesis²⁶. Interestingly, Chu-Wang and Oppenheim¹⁷ have concluded from their ultrastructural studies that ribosomal disassembly and arrested protein synthesis are common features of natural motoneurone death. Thus, the dibutyl cyclic GMP

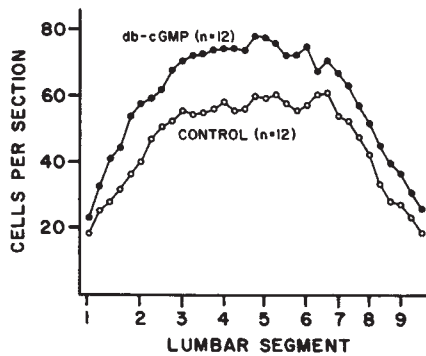


Fig. 2 Mean motoneurone counts per section for both right and left LMC in lumbar segments 1-9 of 10-day control (○) and dibutyryl cyclic GMP (db-cGMP)-treated (●) embryos.

treatment which provides for motoneurone survival also seems to increase the number of nucleoli which, if functional, would result in increased RNA and protein synthesis. We are not aware of any other reports linking cyclic GMP and increased RNA and subsequent protein synthesis in a causal relationship in post-mitotic cells.

These data and those previously reported¹⁹ suggest the following extension of the model for peripheral target regulation of motoneurone death. Muscle target tissue produces glycoprotein factor(s)²⁷ that may or may not be secreted *in vivo*¹⁹. Association of these factors with extending axon growth cones would, either directly or indirectly via axoplasmic transport, result in an increase in cyclic GMP levels in the neurone soma. In the experiments described here, the presence of dibutyryl cyclic GMP circumvents the requirement for the muscle factor(s). Ultimately, either sustained or new protein synthesis would provide for motoneurone survival. It may therefore be more appropriate to consider motoneurone death as inevitable in the absence of appropriately timed intracellular maintenance signals that are required for survival and are only provided by the availability of appropriate target factors.

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Splitting the bithorax complex of *Drosophila*

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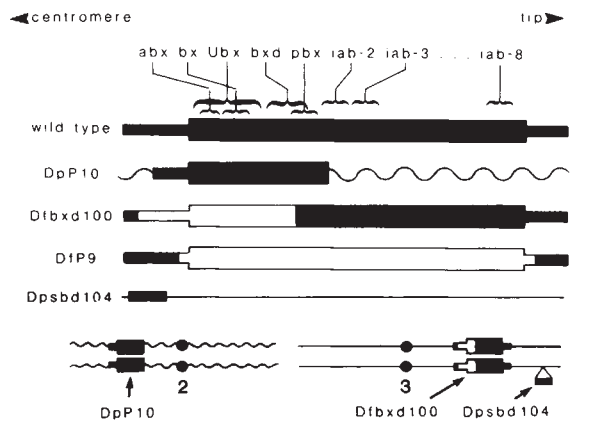
The bithorax complex (BX-C) of *Drosophila* is a series of adjacent genes which control the development of most of the thoracic and abdominal segments¹⁻⁷. Each of these genes is normally active only in particular sets of segments. Moreover, they are ordered along the chromosome in almost exactly the same sequence as that of the segments in which they act. This remarkable correspondence has led to the suggestion^{1,2,5,7} that the progressive unfurling of BX-C activity along the body axis is dictated in part by its genetic organization, and hence, that it may depend on the structural integrity of the entire complex. This suggestion is supported by several cases of 'polar' complementation behaviour observed between mutations in separable genes of the complex¹⁻⁶. Here I show that it is possible to split the complex into two pieces without affecting the development of the larva or adult. This result establishes that the complex is composed of at least two autonomous domains. Surprisingly, these domains do not control the development of separate sets of segments. Rather, their realms of action appear to intersect at the anteroposterior compartment boundary located in the middle of the first abdominal segment. This result extends previous findings⁸⁻¹¹ that anteroposterior compartment boundaries within segments may demarcate the limits of BX-C gene function. Indeed, the phenotypes of larvae carrying only the proximal or distal domain suggest that the BX-C genes act not on segments as wholes, but on segmental units bounded by the anteroposterior compartment boundaries subdividing each segment.

Three chromosomal rearrangements (*T(2;3)P10*, *Tp(3;3)-bxd*¹⁰⁰ and *Tp(3;3)sbd*¹⁰⁴; refs 4, 12, 13) have been used to generate a fertile stock of homozygous flies in which the BX-C complex is split into a centromere-proximal fragment translocated to the left arm of the second chromosome and a centromere-distal fragment which remains at its normal location (see Fig. 1). I am unable to distinguish larvae and adults of this stock from larvae and adults of wild-type stocks by any consistent morphological criterion. Thus, splitting the BX-C as described in Fig. 1 does not appear to alter the development of the larvae or adult, and hence, does not appear to alter significantly the wild-type function of the complex.

The finding that the BX-C can be split into proximal and distal fragments which together dictate normal segmental development indicates that the complex is composed of at least two functionally autonomous domains. To explore the independent roles of these domains, I have examined the segmental phenotypes of first instar larvae which carry only one or the other domain.

The results shown in Figs 2 and 3 extend previous descriptions⁴⁻⁶ of the larval phenotypes and together with studies of similar mutant genotypes in the adult^{1,2,8-11,14,15} allow the following general descriptions of animals carrying only the proximal or distal BX-C domain. The phenotypes of these animals are best described if we consider the body to be composed not of a sequence of integral thoracic and abdominal segments (for example, ... T2, T3, A1, A2, ...) but rather as a sequence of segmental units each composed of the posterior half of one segment and the anterior half of the next segment (for example, ... T1p+T2a, T2p+T3a, T3p+A1a, A1p+A2a, ...) as illustrated in Fig. 4. Animals which carry only the proximal BX-C domain (for example, *DpP10*; *DfP9* larvae, Fig. 2, refs 4, 5) appear wild type anterior to the middle of the first abdominal segment; however, posteriorly, they reiterate the

Fig. 1 Construction of the split bithorax complex. The genetic map of the wild-type BX-C^{4,5,6,26} is shown at the top of the figure and the chromosomal rearrangements used^{4,5,12,13} are illustrated below (BX-C material is represented as a thick bar, neighbouring third chromosomal material as thinner bars; second chromosomal material as a wavy line; distant third chromosomal material as a straight line; deleted regions are shown as white boxes). Three chromosomal rearrangements have been used to generate the split complex. The first, *DpP10*, is an insertional translocation of a centromere-proximal fragment of the complex to chromosome 2. This fragment carries wild-type alleles of the *abx*, *bx*, *Ubx*, *bxl* and *pbx* genes and probably a defective portion of the *iab-2* gene. The second, *Dfbxd¹⁰⁰*, is a deletion of a centromere-proximal fragment which removes the *abx*, *bx* and *Ubx* genes and part of the *bxl* gene. The remaining distal fragment therefore carries part of the *bxl* gene, the *pbx* gene, and all of the *iab* genes; however, neither the *bxl* gene nor the *pbx* gene appear to be functional. Animals homozygous for both the proximal *DpP10* fragment and the distal fragment not deleted by *Dfbxd¹⁰⁰* normally die just before or soon after hatching as first instar larvae because they lack a small region of chromosome 3 which is deleted by *Dfbxd¹⁰⁰* but not carried by *DpP10*. The third chromosomal rearrangement, *Dpsbd¹⁰⁴*, carries a portion of chromosome 3 including this small region to a more distal site on the same chromosome arm. *Dpsbd¹⁰⁴* carries no BX-C material; hence *DpP10; Dfbxd¹⁰⁰* animals carrying at least one copy of *Dpsbd¹⁰⁴* should survive and be phenotypically wild type unless the split in the BX-C causes a mutant phenotype. The chromosomal constitution of flies homozygous for the split complex is shown at the bottom. A fourth aberration, *DfP9*, which deletes the entire BX-C is also shown.

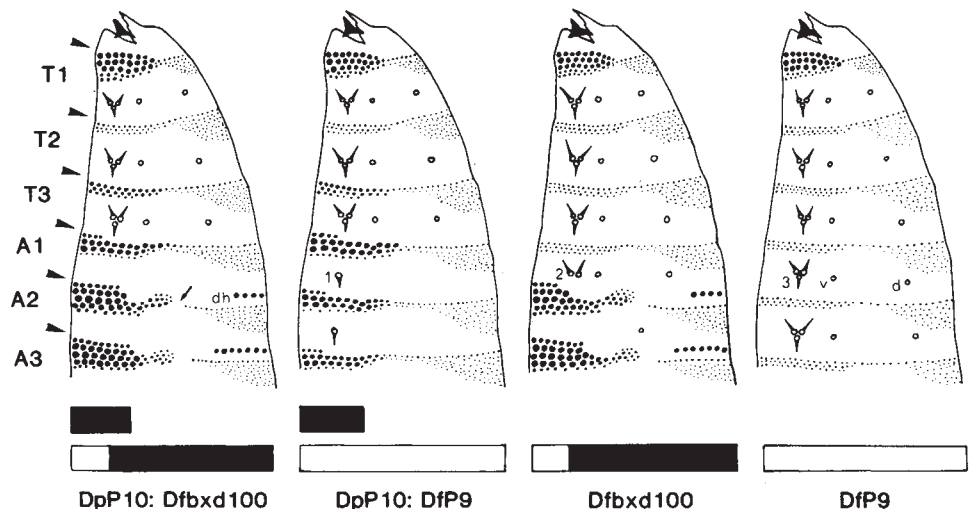


T3p+A1a segmental pattern. Conversely, animals which carry only the distal BX-C domain (for example, *Dfbxd¹⁰⁰* larvae, Figs 2, 3 and refs 4, 5; *Ubx/+* adults carrying *Ubx/Ubx* clones, refs 1, 2, 8–11) approximate the wild-type pattern posterior to the middle of the first abdominal segment, but anteriorly, the T2p+T3a and T3p+A1a units develop as T1p+T2a units.

To understand these seemingly complex phenotypes it may be helpful to consider a simplified version of Lewis's model of the BX-C^{4,5,15,16}. The gist of this model is that the complex is composed of a series of genes (arbitrarily numbered

0, 1, 2, ... 8) which specify segmental state in a successive and cumulative fashion. None of the genes is posited to be active in T2 or more anterior segments. Beginning in T3, gene 0 is active, in A1, genes 0 and 1 are active, in A2, genes 0, 1 and 2 are active, and so forth until A8 in which all the genes are active. For the sake of argument, assume that the proximal BX-C domain defined in Fig. 1 encodes genes 0 (= *Ubx*) and 1 (= *bxl*), and that the distal domain encodes genes 2–8 (= *iab-2-iab-8*)^{4,5}. As shown in Fig. 4, the model predicts that larvae carrying only the proximal domain would appear wild

Fig. 2 Semi-schematic drawings of the lateral aspects of first instar larvae homozygous for the split BX-C (*DpP10; Dfbxd¹⁰⁰*), the proximal fragment (*DpP10; DfP9*), the distal fragment (*Dfbxd¹⁰⁰*), or a deletion of the entire complex (*DfP9*) (see Fig. 1 for descriptions of genotypes; *DpP10; Dfbxd¹⁰⁰* and *Dfbxd¹⁰⁰* larvae also carry *Dpsbd¹⁰⁴*). Arrowheads mark the approximate anterior margins of the thoracic (T1, T2, T3) and first three abdominal (A1, A2, A3) segments. Ventral and dorsal hairs are shown as dots of varying size according to their thickness and length (ventral side left). Partial and complete Keilin's organs (mono-hairs, 1; di-hairs, 2; and tri-hairs, 3), and dorsal (d) and ventral (v) pits are illustrated as indicated in A1. *DpP10; Dfbxd¹⁰⁰* larvae appear indistinguishable from wild-type larvae. Note: (1) that each of the segments bears a unique pattern of ventral and dorsal hairs (though because the posterior portions of the three thoracic segments are bald, they cannot be readily distinguished from each other); (2) that Keilin's organs, and dorsal (d) and ventral (v) pits are formed exclusively in the thoracic segments; (3) that a row of thick dorsal hairs (dh) is formed near the posterior margin of each abdominal segment; and (4) that there is a discontinuity (arrow) in the hair patterns of A2 and A3 near the dorso-ventral midline. *DpP10; DfP9* larvae appear wild type anterior to the middle of A1. However, a partial Keilin's organ consisting of a mono-hair is sometimes present (45%) in the middle of the segment (in rare cases (<1%) a di-hair is formed), and the posterior row of thick dorsal hairs is absent. Hence, the posterior half of A1 appears to develop like the posterior half of a thoracic segment (probably T3). The next several segments (A2–A7) appear identical to A1 (that is, they appear as A1-anterior:T3-posterior). *Dfbxd¹⁰⁰* larvae appear wild type anterior to T3. However, (1) T3 appears like T2; (2) the anterior half of A1 appears like that of T2; (3) the posterior half of A1 appears wild type; and (4) the next several abdominal segments approximate the wild-type pattern. Note that dorsal and ventral pits are always formed in A1 and a partial Keilin's organ consisting of a di-hair is generally formed in the middle of the segment (95%; in the remaining cases, a third hair is also present). Note also that the next several abdominal segments (A2–A7) are not phenotypically wild type, though they approximate the wild-type pattern. In particular, they tend to display abbreviated patterns of ventral hairs suggesting that they have attributes of more anterior abdominal segments (see Fig. 3); in addition, they each generally bear a sensory organ resembling the ventral pit near the dorso-ventral midline and a second sensory organ (not shown) resembling the dorsal pit near the dorsal midline. *DfP9* larvae appear wild-type anterior to T3; however T3 and the first several abdominal segments (A1–A7) appear like T2. These results agree with and extend previous descriptions^{4,5} of the phenotypes of larvae of similar genotype, and are documented more fully elsewhere^{15,16,27}. Larvae were prepared for microscopic inspection by dechorionating and peeling from the vitelline membrane (when necessary), fixing in 1:4 glycerol:acetic acid (60 °C, 30 min)²⁸, rinsing in lactic acid, and mounting in a 1:1 mixture of Hoyer's mountant²⁸ and lactic acid.



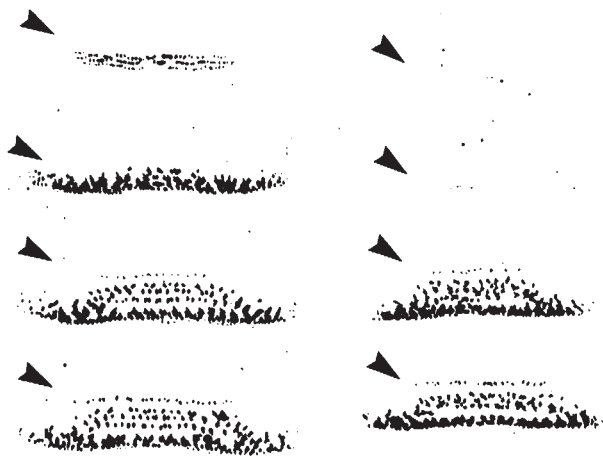


Fig. 3 Photomicrographs of the ventral hair patterns in *DpP10; Dfbxd¹⁰⁰* (a) and *Dfbxd¹⁰⁰* (b) larvae (dark field optics, negative image). Arrowheads mark from top to bottom the approximate anterior margins of T3, A1, A2 and A3. Note that T3 and A1 of the *Dfbxd¹⁰⁰* larva display a pattern of fine hairs characteristic of T2, and hence are not apparent in the photograph. Note also that the hair patterns of A2 and A3 appear abbreviated in *Dfbxd¹⁰⁰* larvae relative to those of *DpP10; Dfbxd¹⁰⁰* larvae (which are indistinguishable from wild type). This difference can be seen most clearly by comparing the number of ventral hairs in the anterior-most rows of each segment (for example, A2 and A3 carry respectively 12 ± 4 and 21 ± 3 hairs in *Dfbxd¹⁰⁰* larvae and 21 ± 2 and 24 ± 3 hairs in *DpP10; Dfbxd¹⁰⁰* larvae; $n = 10$). Magnification $\times 200$.

Fig. 4 Conventional and 'out of register' models of the BX-C. Upper panel (conventional model): this model is a simplified version of Lewis's model (see refs 4, 5, 15, 16) and is illustrated by a set of four matrices in which the anterior (A; unshaded) and posterior (P; shaded) compartments of each segment are rows and the *Ubx*, *bxd*, *iab-2*, and *iab-3* genes are columns. Each matrix shows the expected pattern of active (●) and inactive (○) genes in animals of the genotype indicated at the bottom. According to this model, the level of development of each segment is specified by the particular combination of active and inactive BX-C genes. As shown in the *DpP10; Dfbxd¹⁰⁰* matrix (equivalent to wild type), all of the genes are normally inactive in the mesothorax, but are turned on one by one in each succeeding segment according to their proximodistal order along the chromosome. In the remaining three genotypes, some or all of the genes are deleted; hence the 'code' of active genes is altered in some of the segments. In most cases, the predicted segmental phenotypes (shown to the right of each matrix) are simply the expected outcome of expressing the correct (or sense²⁹) code for one segment in another segment. However, in two cases (A2 and A3 of *Dfbxd¹⁰⁰* animals), an abnormal (or nonsense²⁹) code of active genes is expressed. Such nonsense codes might be expected to specify segments of novel or intermediate character ('(A2)', '(A3)'). Lower panel ('out of register' model): this model is identical to the conventional model above except that the realms of action of the BX-C genes are segmental units which are out of register by a compartment from the segments themselves. For example, the code '●●○○' is normally active in the segmental unit T3p+A1a; if this code is expressed in a different segmental unit (for example, A1p+A2a or A2p+A3a) these units will be transformed into the T3p+A1a unit (as in *DpP10; DfP9* larvae). As in the upper panel, the segmental phenotypes which are predicted by this model are shown to the right of each matrix. Note that they are in good agreement with the larval phenotypes described in Figs 2 and 3, and elsewhere^{4,5,16,30}, and with the adult phenotypes caused by equivalent mutations or chromosomal rearrangements^{1,2,8-11,14,15}. Because the posterior compartments of the thoracic segments appear indistinguishable from each other in wild-type larvae, their segmental status cannot be readily assessed without recourse to studies of the adult compartments. Fortunately, the adult phenotypes of mutations or chromosomal aberrations which inactivate (1) the *Ubx* and *bxd* genes⁹⁻¹¹, (2) the *bxd* gene^{1,2,8,14}, and (3) the *iab-2* and *iab-3* genes¹⁵ have been studied, and in most cases, the segmental transformations observed in the posterior compartments of the thorax and abdomen are in close accord with the transformations predicted by the model. Two exceptions should be noted. First, *bxd* flies often form legs and occasionally wing-like halteres composed of both anterior and posterior compartments in place of the first abdominal segment^{1,2,14} (though possibly in place of only the anterior compartment^{11,14}). Second, *Ubx* mutations do not appear to affect the posterior wing compartment even though they transform the posterior compartment of the second leg to posterior first leg^{9,10}. Further study of these exceptional cases will be necessary to assess the validity of the 'out of register' model.

type in all segments anterior to A2; however, posteriorly, they would reiterate the A1 pattern. Conversely, it predicts that animals carrying only the distal domain would approximate the wild-type pattern in segments posterior to A1, but anteriorly, T3 and A1 would develop as T2. Comparing these simple predictions with the actual phenotypes described in Figs 2 and 3 and refs 1, 2, 4, 5, 8-11, 14-16 (summarized above), it becomes apparent that they are out of register with the actual results by half a segment. As illustrated in Fig 4, this discrepancy can be resolved if one merely shifts the stipulated realms of action of the BX-C genes anteriorly by half a segment. This resolution suggests that the BX-C genes may normally act on segmental units extending from the middle of one segment to the middle of the next.

If this interpretation is correct, one might ask why the boundaries demarcating the realms of action of the BX-C genes fall in the middle of the segments. One possible explanation is that these border lines coincide exactly with the strict lineage boundaries that subdivide each segment into an anterior and posterior compartment¹⁷⁻²³. This explanation is suggested by the demonstration that some BX-C mutations clearly transform one compartment of an adult segment without altering the remaining compartment⁸⁻¹¹ and is supported by the particular kinds of incomplete Keilin's organs formed in the first abdominal segment of larvae lacking either the proximal or distal BX-C domain (Fig. 2; refs 4,5).

Keilin's organs (or tri-hairs) are normally found only in the thoracic segments, though in the absence of the entire BX-C they also appear in the first eight abdominal segments which develop like thoracic segments. In larvae lacking the proximal

Prothorax	A			T1			T1			T1			T1
	P												
Mesothorax	A		0000	T2		0000	T2		0000	T2		0000	T2
	P												
Metathorax	A		●000	T3		●000	T3		0000	T2		0000	T2
	P												
Abdominal 1	A		●●00	A1		●●00	A1		0000	T2		0000	T2
	P												
Abdominal 2	A		●●00	A2		●●00	A1		0000	(A2)		0000	T2
	P												
Abdominal 3	A		●●●●	A3		●●00	A1		000●	(A3)		0000	T2
	P												

Prothorax	A			T1			T1			T1			T1
	P												
Mesothorax	A		0000	T1		0000	T1		0000	T1		0000	T1
	P			T2			T2			T2			T2
Metathorax	A		●000	T2		●000	T2		0000	T1		0000	T1
	P			T3			T3			T2			T2
Abdominal 1	A		●●00	T3		●●00	T3		0000	T1		0000	T1
	P			A1			A1			T2			T2
Abdominal 2	A		●●●●	A1		●●00	T3		0000	(A1)		0000	T1
	P			A2			A1			(A2)			T2
Abdominal 3	A		●●●●	A2		●●00	T3		0000	(A2)		0000	T1
	P			A3			A1			(A3)			T2
	P			A3			T3			(A3)			T1

Ubx	bxd	iab-2	iab-3
●	●	●	●
○	○	○	○

			
			
DpP10; Dfbxd100	DpP10; DfP9	Dfbxd100	DfP9
●●○○	●●○○	○○○○	○○○○

domain, the anterior half of the first abdominal segment develops like the anterior mesothorax and incomplete Keilin's organs composed of di-hairs are generally found in the middle of the segment. Conversely, in larvae lacking the distal domain, the posterior half of this segment develops like the posterior metathorax and mono-hairs are sometimes found in the middle of the segment. The reciprocal nature of these phenotypes suggests that the Keilin's organs which form in the first abdominal segment of BX-C⁻ larvae may be mosaic structures consisting of two hairs which are generally formed by cells in the anterior half of the segment, and a third which is sometimes formed by cells in the posterior half.

In his comparative studies of the morphologies of Dipteran larvae^{24,25}, Keilin established that the 'bouquet' of hairs constituting the 'organes sensories vestigiaux des pattes' are positioned exactly at the attachment sites of the imaginal leg disks in each thoracic segment, and are contiguous with the disk stalk. Indeed, for this and related reasons, he proposed that they are evolutionary vestiges of the larval legs generally absent in the Diptera. Because (1) the imaginal leg disks are subdivided into anterior and posterior compartments^{20,21}, and (2) this compartmental segregation probably extends down the stalk of the disk and subdivides the adjacent larval epidermis into anterior and posterior compartments²², it is possible that the Keilin's organ straddles the compartment boundary. Hence, two of its three hairs may generally derive from cells in the anterior compartment (as in the first abdominal segment of larvae lacking the proximal BX-C domain) whereas the third hair may sometimes derive from cells in the posterior compartment (as in the first abdominal segment of larvae lacking the distal BX-C domain). Thus, the realms of action of the BX-C genes in the proximal and distal domains may intersect precisely at the antero-posterior compartment boundary of the first abdominal segment, extending previous findings⁹⁻¹¹ that the realms of action of at least some BX-C genes are demarcated not by segment boundaries, but rather by anteroposterior compartment boundaries within segments.

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Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses

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We describe here a novel type of immunostimulating complex, called 'iscom', in which virus membrane proteins are presented in a multimeric form¹⁻⁸. The matrix of the iscom is the glycoside Quil A (Spikoside; Iscotec AB), extracted from the bark of *Quillaja saponaria* Molina⁹, which forms micelles at the critical micellar concentration of 0.03%. In micelle form, Quil A probably has regions accessible for hydrophobic interaction with the membrane proteins so that it can form complexes with them. Iscoms have been prepared with membrane proteins of parainfluenza-3 (PI-3), measles and rabies viruses, and their immunizing potency tested in animals. In these experiments, iscoms prove to be at least 10 times more potent than micelles formed by aggregation of the membrane proteins alone⁴. Iscoms of PI-3 and measles viruses also stimulate the formation of antibody to the fusion (F) protein, which is considered to be poorly immunogenic^{10,11}. No side effects of iscoms or of protein micelles have been observed.

Initially, parainfluenza-3 (PI-3) virus was chosen since we had previously used its envelope glycoproteins in experiments with a micelle vaccine^{3,12}. The glycoproteins were prepared by centrifugation of the solubilized virus layered on a sucrose gradient containing 0.2% Quil A. The details of this procedure are given in Fig. 1. The protein micelles were prepared by sucrose gradient centrifugation as described previously³.

An iscom is characterized by its sedimentation coefficient, protein profile on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by electron microscopy. In a 10-40% sucrose gradient, the sedimentation coefficient was calculated to be 19S for iscoms¹³ and 30S for the protein micelles, as described elsewhere^{3,12}. The protein pattern of the iscom is similar to that of the protein micelle in SDS-PAGE¹⁴. In this gel two protein bands were seen: the larger one was a protein of molecular weight (MW) 73,000 (73K) and was identified as the haemagglutinin-neuraminidase (HN) protein. The smaller one was a protein with a MW of 51K and is probably the fusion (F) protein^{11,15}.

The two types of complexes—the iscom and the micelle—showed different morphology. The iscoms are particles with a mean diameter of 35 nm. Each particle has a loose, translucent, cage-like structure with ring-like subunits about 12 nm in

Table 1 The immunopotentiating effect of Quil A added with iscoms

Protein (μg) per dose	Quil A (μg) added and HI-titre*		
	0	1	10
0.5	6.8 ± 0.6	6.9 ± 0.5	7.3 ± 0.7
1	7.1 ± 0.4	7.3 ± 0.0	8.1 ± 0.8
5	8.1 ± 0.4	8.3 ± 0.0	9.1 ± 0.4

Five mice per group were vaccinated subcutaneously with three different doses of iscoms prepared with parainfluenza-3 virus envelope proteins twice three weeks apart. The antibody response was measured by the HI titres²⁵.

* Reciprocal of twofold serum dilution in 2 log.

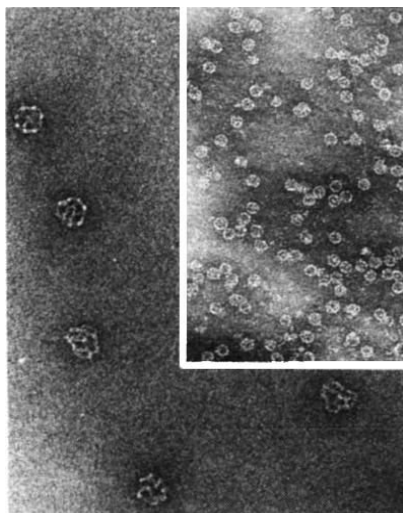


Fig. 1 Electron micrographs of iscoms containing the membrane glycoproteins of parainfluenza-3 (PI-3) virus. PI-3 virus, strain U23B (ref. 26), was purified by sucrose gradient centrifugation as described before¹² and was solubilized with 2% Triton X-100 together with ³H-labelled virus¹⁹ (10⁵ c.p.m.) in 0.05 M Tris buffer and 0.1 M NaCl (TN). Two hundred μ g of solubilized virus in 100 μ l TN was applied onto a layer of 200 μ l 8% sucrose in TN and 1% Triton X-100 which was layered over a 5 ml sucrose gradient in TN ranging from 10 to 40% and containing 0.2% Quil A. The centrifugation was performed in a SW50 rotor at 150,000g for 4 h at 20 °C. Fractions of 250 μ l were collected and measured for radioactivity. The radioactive peak fractions were pooled and dialysed for 2 days at 4 °C against 3 $\times$ 1 l of 0.05 M ammonium acetate, pH 7.0. The pooled fractions were concentrated by lyophilization. The virus protein concentration was determined by the Lowry method²⁷. The amount of Quil A in an iscom preparation was measured by an electrophoresis lysis method¹⁸ to $\leq$ 2 μ g per 10 μ g protein. $\times$ 105,000; insert, $\times$ 40,000.

diameter (Fig. 1). The cage-like structure appears somewhat flattened as judged from tilted or shadow-cast specimens; the structure has some similarities to cage-like clathrins¹⁶. It is interesting that the stain penetrates into the iscom as it does into incomplete, nucleic-acid free virus capsids of adenovirus¹⁷. The micelle has a characteristic hydrophobic dense core with protruding hydrophilic parts (not shown).

How then are the iscom particles formed? Our hypothesis was that, at equivalence, Quil A micelles capture the monomer forms of the envelope proteins sedimenting from the detergent layer of the sucrose gradient, which then bind to the surface of the Quil A micelles by hydrophobic interaction. The data suggest that the micelle form of Quil A is necessary for the formation of iscoms. The concentration of Quil A had to be at or above the critical micelle concentration of 0.03%, with a protein concentration of 100–500 μ g in the virus sample. In the immunization studies described below the amount of Quil A was estimated as 2 μ g per 10 μ g of protein by a lysis method described elsewhere¹⁸. After re-centrifugation of iscoms in a sucrose gradient prepared in an isotonic Tris buffer at pH 7.2, no Quil A was detected by the lysis method. Quil A labelled with the galactose oxidase ³H-borohydride method¹⁹ was used to determine its amount bound within the iscoms. Measuring the radioactivity in iscoms purified in a sucrose gradient, the amount of Quil A was calculated to about 2 μ g per 10 μ g protein.

The haemagglutinin of PI-3 virus is externally exposed on the iscom as iscoms, like micelles, agglutinated guinea pig erythrocytes in protein concentrations 10–30 times lower than those of intact virus particles. The haemagglutination was specifically inhibited by rabbit antiserum to PI-3 virus glycoproteins.

We have examined the immunological potency of iscoms in vaccination experiments on BALB/c mice by comparing iscom and protein-micelle vaccines. The potency was measured by

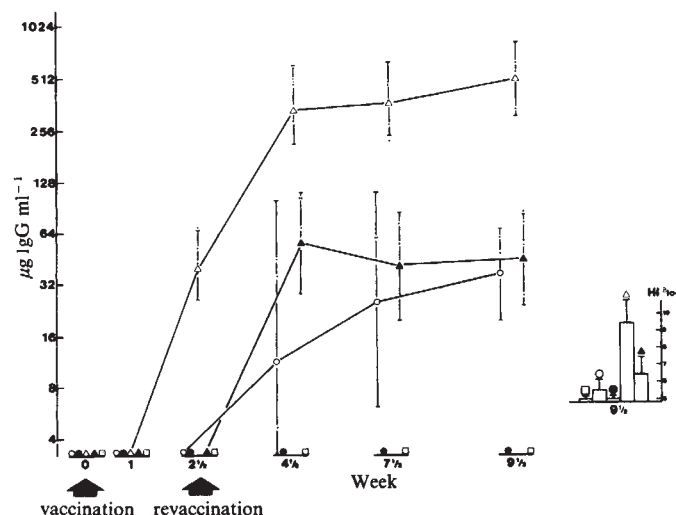


Fig. 2 The antibody response of BALB/c mice to vaccination with PI-3 virus protein micelles or iscoms. $\circ$, 5 μ g of protein micelles; $\bullet$, 0.5 μ g of protein micelles; $\triangle$, 5 μ g of iscoms; $\blacktriangle$, 0.5 μ g of iscoms; $\square$, non-vaccinated mice. Symbols indicate mean values, dots individual sera, and vertical bars the standard deviation. Ten mice per vaccination group were kept in cages of five. They were vaccinated twice 2.5 weeks apart with 5 or 0.5 μ g of PI-3 virus envelope proteins as protein micelles or iscoms. Blood samples were collected by bleeding the mice from the retrobulbar plexus. Five mice from each vaccination group were bled at the time. Serum was prepared and stored at -20 °C until used. The immune response was measured in an enzyme-linked immunoassay (ELISA) as before³. The specific IgG response to the virus proteins was quantitated as described by McLean *et al.*²⁸. At the end of the experiment the HI titre was determined²⁵.

antibody titres in serum. First the adjuvant effect of free Quil A present in the iscom and micelle preparations was tested (Table 1). The results show that significant rises of haemagglutination inhibition (HI) titres ($P \leq 0.0025$) were induced by addition of 10 μ g Quil A, which is in accord with the previous results of Dalsgaard (manuscript in preparation). Then the immune response to iscoms as well as protein micelles was tested (Fig. 2). The results allow the following conclusions. First, measured by two different techniques the iscoms induced 10 times higher antibody response than the micelles. Iscoms with 5 μ g protein per dose induced the production of specific IgG of up to 500 μ g ml⁻¹ serum. Second, to induce an antibody response of similar magnitude 10 times more antigen had to be included in the micelles. Third, a single vaccine dose of iscoms induced about the same antibody response as two doses of micelles with the same amount of antigen per dose.

To study whether antibody to both the glycoproteins of PI-3 virus is induced, an immunoelectroblotting experiment was performed. Sera from mice vaccinated either with iscoms or micelles reacted solely with the HN and F proteins (Fig. 3), whereas whole killed paramyxoviruses induce antibodies to several other virus proteins^{10,11}, including the HN but not the F protein. Iscoms were then prepared with these spike proteins of measles virus (H and F), another member of the Paramyxoviridae family, and tested for their antigenicity in laboratory rats. The rats were inoculated once intramuscularly with 5 μ g of antigen. In the same experiment two other groups of rats were included which were immunized with β -propiolactone (BPL)-killed measles virus or protein micelles both preparations containing 5 μ g or the spike proteins per dose. The results in Fig. 4 show that the iscoms not only efficiently induce anti-H antibody measured with a HI test and virus neutralizing antibody but also antibody to the F protein as measured in a haemolysis inhibition (HLI) test, which is in contrast to killed virus and micelles (not shown). Since both iscoms and micelles were prepared from BPL-killed virus it appears that the presentation of the antigen is responsible

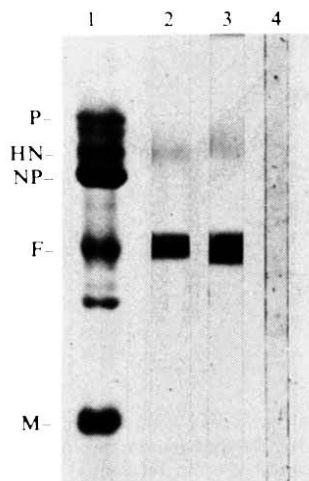


Fig. 3 Antibodies to HN and F proteins of PI-3 virus detected by immunoblotting in the sera from mice vaccinated with these proteins as iscoms or micelles. Purified PI-3 virus was submitted to 10% SDS-PAGE gels in SDS. The gel was transferred to nitrocellulose sheets by the method of Towbin *et al.*²⁹. The sera were obtained from mice vaccinated twice 4 weeks apart with 5 µg PI-3 virus membrane glycoproteins prepared as iscoms (serum A) or as micelles (serum B). The sera were obtained 8 weeks after the second vaccination. The immunoblotting was performed as follows. The nitrocellulose sheet was preincubated in phosphate-buffered saline (PBS), pH 7.4, 10 µg ml⁻¹ bovine serum albumin (BSA), 0.2% Triton X-100 for 16 h at room temperature. Twenty µl of mouse serum was diluted with 1 ml PBS, pH 7.4 containing 5 mg of BSA and 0.2% Triton X-100. The mixture was incubated on the nitrocellulose strips for 1 h at room temperature with a rabbit anti-mouse IgG peroxidase conjugate (Dakop, Denmark) diluted 1/100. The cellulose strips were washed in five changes of PBS, pH 7.5 and the peroxidase conjugate was localized by dipping the strips in the substrate 3-amino-9-ethyl cabazole (Sigma, Saint Louise country) dissolved in buffer pH 5.4 containing 0.01% H₂O₂. Lane 1, Coomassie brilliant blue stained PI-3 virus; lane 2 developed with serum from mice immunized with micelles; lane 3 developed with serum from mice immunized with iscoms; lane 4, developed with serum from non-vaccinated mice.

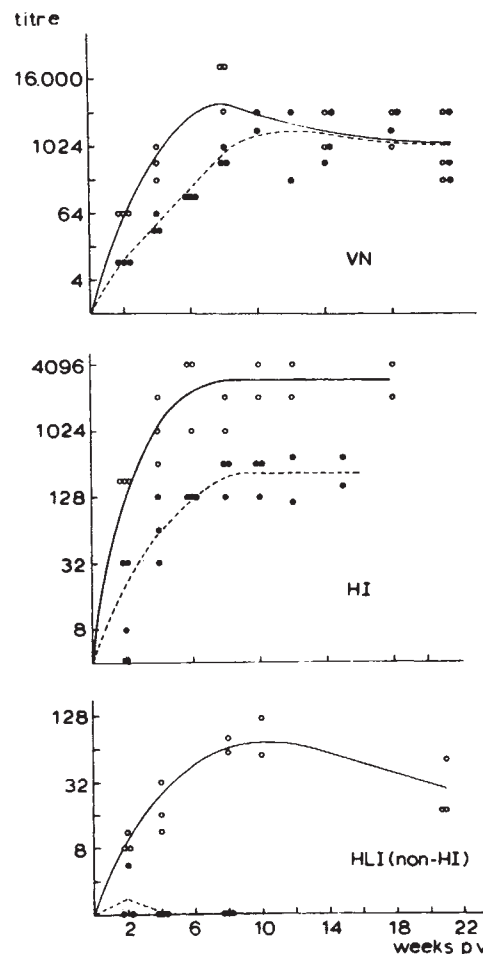


Fig. 4 Antibody response of two groups of three Wistar rats to a single intramuscular vaccination with BPL-killed measles virus or with measles virus iscoms. Titres were measured in a virus neutralization (VN) test, a HI test, and a HLI test after absorption with Tween-ether treated measles virus antigen as described by Norrby *et al.*¹⁰. The symbols indicate values of individual animals vaccinated with: ●, BPL killed virus containing 5 µg of glycoprotein per dose; ○, measles virus iscoms containing 5 µg of glycoprotein per dose. Blood samples were collected by cardiac puncture and serum was stored at -20 °C until use.

for the differences in response between the different preparations.

Finally, a rabies iscom vaccine was tested in the NIH test for potency of rabies vaccines using the third WHO reference vaccine preparation (1978) as standard. One vaccine dose containing 4.5 µg of rabies virus glycoprotein corresponded with an antigenic value of 1.0 (ref. 20) that is, it induced the same protective immunity in mice as one whole dose of the reference vaccine.

Why then are the iscoms more efficient than a micelle vaccine in evoking antibody response? As seen in Fig. 1 the morphology of the iscom particle is different from that of the protein micelle in that in the iscom a larger part of the spikes is exposed. Another explanation for the efficacy of the iscoms is that Quil A itself exerts an adjuvant effect similar to that shown with other antigens²¹⁻²³. However, in the iscom the amounts of Quil A were too low to exert an adjuvant activity like that in conventional vaccines⁹. It is not known whether Quil A linked to the antigens may exert adjuvant activity at lower concentrations. However, it has been reported that muramyl dipeptide had adjuvant activity in much lower concentrations when covalently linked to a synthetic antigen²⁴. In the iscom vaccine containing 0.5 µg antigen per dose, the amount of Quil A was 50-fold lower than that required to obtain conventional adjuvant activity in mice (K.D., in preparation).

In conclusion, as a vaccine, the iscom has the advantage that it can be standardized biochemically with respect to the antigens and their physical form, and after administration it appears to cause no side effects. The iscom seems to be reasonably stable

since in preparations purified from unbound Quil A it retained the 19S value as well as its characteristic morphology several months after lyophilization and reconstitution. Thus, the iscom might be an interesting new form of a subunit vaccine.

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Therapeutic potential of monovalent monoclonal antibodies

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One therapeutic use for monoclonal antibody technology¹ is the elimination of categories of unwanted cells by virtue of their distinct cell surface antigens. The efficiency of cell destruction by complement lysis or opsonization depends on a number of factors such as antibody specificity and isotype as well as certain properties of the target antigen². In some instances cells can escape destruction by redistributing and eventually losing the antigen-antibody complexes from their surface^{3,4}. This process, known as antigenic modulation, generally depends on bivalent antibody binding. Starting from the observation that rabbit antisera can be made more effective at killing tumour cells if they are first rendered univalent by limited proteolysis, we have now prepared a number of monovalent rat monoclonal antibodies to human cell-surface antigens. We find that these antibodies are no longer able to bring about modulation of their target antigens and have an enhanced facility for lysis with human complement. These special properties should greatly increase the therapeutic potential of monoclonal antibodies.

We have produced a number of rat-rat hybrid myelomas secreting antibodies against mouse or human haematopoietic cell surfaces by fusing DA rat spleen cells to the Y3/Ag1.2.3 rat myeloma line⁵. All the hybrids were found to secrete both the specific antibody light chain from the DA rat and the light chain from the Y3/Ag1.2.3 myeloma. If a random association of equal proportions of the two light chains is assumed there will be three antibody species in the culture supernatants: (1) bivalent active immunoglobulin with two specific DA rat light chains; (2) bivalently inactive immunoglobulin with two myeloma-derived light chains; and (3) monovalent immunoglobulin with one Y3/Ag1.2.3 light chain and one DA rat light chain. In all cases, the Fc region remains unaffected and therefore able to interact with complement or other effector systems. The bivalently active immunoglobulin was removed by selecting only those molecules containing the LOU (κ 1a) light-chain allotype of the Y3/Ag1.2.3 line by immunoadsorption using the monoclonal antibody MRC OX 12 coupled to Sepharose as described in Fig. 1.

It is difficult formally to prove the monovalency of the antibody preparations selected in this way, partly because of contamination by a variable amount of the binary inactive material, although with appropriate anti- κ 1b affinity purification this could be removed. We, therefore, studied a range of monoclonal

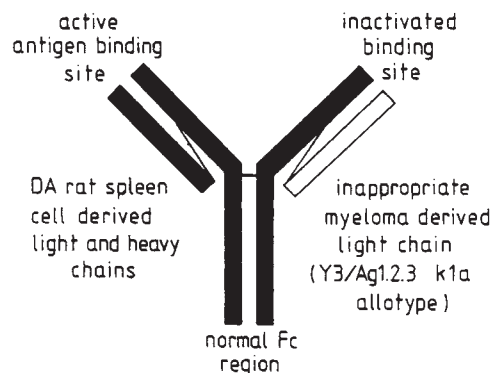


Fig. 1 Hybrid molecules containing light chains derived both from DA rat spleen (κ 1b allotype) and the myeloma Y3/Ag1.2.3 (κ 1a allotype) were separated from bivalently active molecules (see text) by means of the mouse monoclonal antibody MRC OX 12¹⁷ (a gift of Drs S. Hunt and A. Williams), which binds preferentially to the κ 1a allotype¹⁸ of the myeloma light chain. MRC OX 12 antibody was first purified by affinity on a Sepharose column carrying rat immunoglobulin and then coupled to CNBr-activated Sepharose (Pharmacia, manufacturer's recommendations) to give 8 mg pure antibody and 2 mg BSA per g of dry gel. This material bound effectively⁷ only κ 1a light chain containing monoclonal antibodies. Culture supernatants from DA x Y3/Ag1.2.3 hybrid myelomas⁴ were precipitated with 50% saturated $(\text{NH}_4)_2\text{SO}_4$ and incubated with the MRC OX 12-Sepharose in double strength phosphate-buffered saline (PBS) for 3 h at 4°C using ~2 mg protein ml⁻¹ of wet gel material. After removal of unbound material, the column was washed with 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl. Adsorbed antibody containing the monovalent mixed antibodies was eluted with 1 M glycine/HCl pH 2.5 and neutralized with Tris base. This was precipitated with 50% saturated $(\text{NH}_4)_2\text{SO}_4$ and dialysed into PBS, microfuged at 14,000 r.p.m. for 5 min, and the protein estimated by the absorbance at 280 nm. Yields were in the range 50-100 μ g of protein per ml of packed MRC OX 12-Sepharose. BSA was added to 0.5% and the antibodies were stored frozen at -20°C or -70°C. All antibody preparations were centrifuged for 15 min at 160,000g (Beckman Airfuge) before use. To monitor the affinity purification, monovalent and unfractionated preparations were compared for the proportion of antibody containing κ 1a or κ 1b light chains. All of the antigen-binding activity could be removed by a monoclonal antibody to the κ 1b allotype (RG-11/15.5)¹⁹ adsorbed to plastic microtitre trays or coupled to Sepharose (although activity could not be eluted). This demonstrates that the Y3/Ag1.2.3 (κ 1a) light chain does not contribute to antigen binding. On the other hand, adsorption of YTH 12.5.22 and YTH 3.2.6 on MRC OX 12 (anti- κ 1a, in the presence of excess DA rat (κ 1b) serum to ensure specificity) removed only 15-20% and 22-55% respectively of the antigen-binding activity in the unfractionated material, but removed all of the activity of the monovalent preparations. These observations show that the MRC OX 12 affinity column has enriched for those immunoglobulin molecules which each carry the two different light chains. Of the total immunoglobulin (as measured by a solid-phase assay for total light chains), ~50-70% of the YTH 12.5.22 and 20-40% of the YTH 3.2.6 monovalent preparations bound to antigen or could be adsorbed by anti- κ 1b coated microtitre trays, implying that the remainder was inactive material including immunoglobulin molecules with two Y3/Ag1.2.3 light chains.

antibodies against cell-surface antigens to determine whether our preparations had the properties predicted for monovalency; that is, an increase in saturation binding with no agglutination or surface antigen redistribution.

Four monoclonal antibodies were chosen (Table 1). The first, YBM 34.3.6, is directed against a heat-stable antigen (HSA) on cells derived from mouse bone marrow⁶ and was chosen because it strongly agglutinates erythroid cells. The monovalent form of this reagent was found to lose the ability to agglutinate while giving increased saturation binding. (Weakly agglutinating aggregates developed on storage, but could be removed by

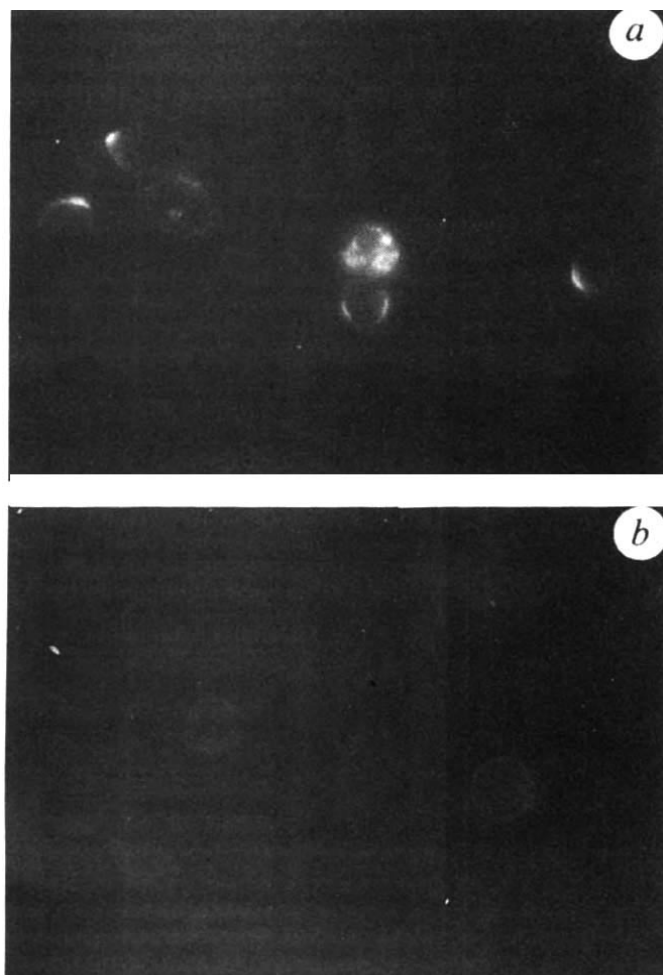


Fig. 2 Monovalent YTH 12.5.22 prepared by the method of Fig. 1, or the whole immunoglobulin fraction from culture supernatant, were conjugated with FITC in 0.1 M bicarbonate buffer pH 9.3. The coupled material was dialysed into PBS and the molar FITC:protein ratio determined according to the absorbances at 493 nm and 280 nm. These were stored at -70°C at 1 mg ml^{-1} in 1% BSA and microfuged (14,000 r.p.m. per 5 min) before use. Human mononuclear cells from defibrinated venous blood were incubated with the FITC-antibodies at $100\text{ }\mu\text{g ml}^{-1}$ in Iscove's modification of Dulbecco's medium (IMDM) containing 0.5% BSA at 37°C for 2 h and then washed. Cells were photographed with Kodak Tri-X on a Leitz microscope ($\times 60$ oil immersion objective) set up for fluorescein excitation and emission. *a*, YTH 12.5.22 unfractionated immunoglobulin-FITC (molar FITC:protein ratio 5.5). 70% of the cells were positive, with $\sim 90\%$ showing redistributed fluorescence, as shown. *b*, YTH 12.5.22 monovalent immunoglobulin-FITC (molar FITC:protein ratio 2.0). 70% of the cells were positive with $\sim 80\%$ of these showing unpatched 'ring' fluorescence, as shown.

centrifugation at $160,000g$ for 15 min.) These properties have been of practical value in the study of antigen expression on bone marrow cells with the fluorescence-activated cell sorter, where agglutination can be a serious problem⁷; the use of biotin-coupled monovalent YBM 34.3.6 with avidin-fluorescein isothiocyanate (FITC) has allowed fluorescence analysis at saturation on total marrow⁷, which had been impossible with previous staining procedures.

The second antibody, YTH 89.1.8, reacts with the glycoporphin-A molecule (G. Hale, personal communication), and can lyse human red cells with human complement. The monovalent preparation of this antibody is at least as effective at killing the cells with complement, and shows increased saturation binding and loss of (weak) agglutinating activity.

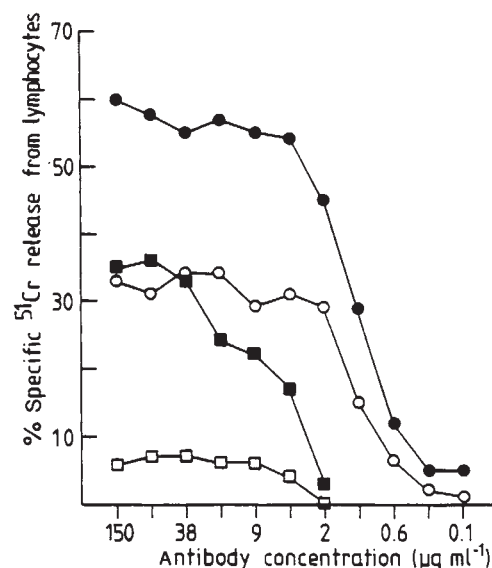


Fig. 3 Human mononuclear cells were prepared by centrifugation on Ficoll-Hypaque, and labelled with ^{51}Na -chromate. 10^5 cells were incubated with $100\text{ }\mu\text{l}$ diluted antibody and $100\text{ }\mu\text{l}$ of either donor human serum or rabbit serum diluted 1/5 (final dilution 1/10) in IMDM with 0.5% BSA in microtitre wells. These were incubated at 37°C for 1 h and the cells then pelleted and the supernatants removed for gamma counting. % Specific ^{51}Cr release from lymphocytes was calculated by the formula:

% Specific ^{51}Cr release

$$= \frac{\text{experimental release} - \text{spontaneous release}}{\text{CAMPATH 1 release} - \text{spontaneous release}} \times 100$$

where CAMPATH 1 is a human and rabbit complement-fixing antibody which kills all lymphocytes¹⁵, and the spontaneous release was from cells incubated without antibody. ●, Monovalent YTH 12.5.22 immunoglobulin plus rabbit complement; ■, YTH 12.5.22, unfractionated immunoglobulin plus rabbit complement; ○, monovalent YTH 12.5.22 immunoglobulin plus human complement; □, YTH 12.5.22, unfractionated immunoglobulin plus human complement.

The other two monoclonal antibodies selected (Table 1) are of the rat IgG2b subclass and are directed against human T cells. One of these (YTH 12.5.22) reacts with a determinant on the T3 molecule (unpublished data). They were chosen because they gave partial lysis with heterologous rabbit complement. However, they gave only poor killing with human complement (serum from the donor of the target cells) even though it has been shown that rat IgG2b antibodies are the most effective of the rat IgG subclasses at fixing human C1q (refs 8–9).

Monoclonal antibodies to the T3 antigen have not been completely effective in eliminating T cells, either by opsonization¹⁰ or by complement lysis¹¹ in treatment for graft-versus-host disease. Similar problems have been met in attempts to use the anti-CALLA monoclonal antibody (J5) to eliminate leukaemic cells *in vitro* and *in vivo*¹², where the antibody has been shown to induce patching and capping of the antigen-antibody complex on the cell surface¹³. By immunofluorescence, both T-cell specific antibodies described here, in their unfractionated forms, gave pronounced redistribution of their target antigens (Fig. 2*a*), while the monovalent preparations gave perfect 'ring' fluorescence in the majority of labelled cells (Fig. 2*b*). Further evidence for the monovalency of the preparations is the increase in maximal binding compared with the supernatant antibody, although neither of these antibodies shows the clear binding plateau obtained with YBM 34.3.6 and YTH 89.1.8.

Even if our novel preparations of monoclonal antibodies behave monovalently, it is by no means obvious that they should still lyse cells with complement, given, for example, that the

Table 1 Properties of monovalent monoclonal antibodies

Monoclonal antibody		Antigen	Isotype	Agglutination of RBC	Maximal binding	Maximal complement kill RC/GPC	Human C	Patching/capping
YBM 34.3.6	Supnt* MONO.†	HSA of mouse BM cell	IgG2c	+++§	0.17	97%	ND	ND
				—	0.28	97%	ND	ND
YTH 89.1.8	Supnt MONO.	Human glycophorin A	IgG2b	(+)	0.18¶	ND	95%	ND
				—	0.27	ND	98%	ND
YTH 12.5.22	Supnt MONO.	Human‡ T cells	IgG2b	—	0.83*	34%**	6%	+++††
				—	1.52	60%	36%	—
YTH 3.2.6	Supnt MONO.	Human T cells	IgG2b	—	0.10*	22%	3%	+
				—	0.20	47%	14%	—

All antibody preparations were microfuged at 14,000 r.p.m. for 10 min before use, and all assays were at room temperature or 37 °C in medium containing 0.5% bovine serum albumin (BSA) and no azide. Abbreviations used: ND, not determined; HSA, heat-stable antigen; BM, bone marrow; RC, rabbit complement; GPC, guinea pig complement; HC, human complement; SPELBA, solid-phase enzyme-linked binding assay.

* $(\text{NH}_4)_2\text{SO}_4$ precipitated antibody from culture supernatants.

† Monovalent mixed light-chain antibody prepared on MRC OX 12-Sepharex.

‡ Blocked by UCHT1 (anti-T3) on T cells, also binds to platelets and null cells.

§ Monovalent YBM 34.3.6. antibody gave no agglutination of mouse red cells up to a concentration of 250 $\mu\text{g ml}^{-1}$ protein whereas original unfractionated YBM 34.3.6 agglutinates at $<1 \mu\text{g ml}^{-1}$.

||, ¶ Change in Δ_{405} in β -galactosidase SPELBA¹⁶ using mouse red blood cells (RBC)||; using human RBC¶; using human T-cell line AK45 (a gift from Dr A. Karpas, Cambridge)*.

** Release of ⁵¹Cr from labelled human peripheral blood lymphocytes expressed as % of release with CAMPATH 1 (ref. 15) which kills all lymphocytes.

†† Assessed using directly coupled FITC-antibody on fluorescence microscope.

avidity of binding would be markedly reduced. However, Fig. 3 shows that the monovalent YTH 12.5.22 is more effective at lysing human peripheral blood T cells with complement than the unfractionated immunoglobulin. (Similar results have been obtained with the other antibody, YTH 3.2.6, see Table 1.) With rabbit serum as the source of complement, the unfractionated antibody gives a plateau kill equivalent to only 34% of the lymphocytes, while with the monovalent fraction, this is improved to 60%, consistent with the proportion of T cells in the peripheral blood. (If the rabbit serum concentration is raised from 1/10 to 1/2, both preparations give ~70% lysis.) When autologous human complement (from the donor of the cells) is used, almost no lysis is observed with the unfractionated antibody (6%). The monovalent YTH 12.5.22, however, is able to kill 36% of the lymphocytes with the human serum which, although insufficient to account for all the T cells, is a significant improvement. It is possible that by manipulation of the complement concentrations and incubation conditions this level of killing could be further improved.

To exclude the possibility that the increase in complement-dependent lysis in the monovalent fractions could have been due to aggregation or some other effect of the processing, unfractionated material was treated in the same way with the low pH buffers and $(\text{NH}_4)_2\text{SO}_4$ precipitation. This did not alter the amount of cell lysis obtained in the presence of complement.

It is clear that monoclonal antibodies which have been enriched for mixed molecules with both specific and myeloma-derived light chains have the predicted properties of monovalent antibodies in terms of their binding to cell surfaces. It is of particular theoretical and practical significance that this novel method of preparing monovalent monoclonal antibodies leads to highly enhanced cytotoxic properties. This could be explained by two main factors, first an increase in the amount of antibody bound to the cell leading to increased Clq fixation¹⁴, and second, a reduction in the redistribution of antigen-antibody complexes on the cell surface, thereby avoiding the effects of antigenic modulation³.

Monoclonal antibodies fixing human complement have a number of potential advantages for therapy¹⁵. For example, *in vitro* they would eliminate any requirement for the often toxic heterologous complement. In two of the examples described

above, a new ability to lyse cells with human complement has been realized by the preparation of monovalent antibodies.

The method of fractionating mixed immunoglobulin molecules has two major advantages over chemical or proteolytic techniques; first, it does not depend on the unusual heavy chain glycosylation required for the enzymatic procedure³, and second, the monovalent mixed molecules are a major proportion of the antibody secreted by the hybrid myeloma, maintaining all the advantages of monoclonal antibody technology. Having demonstrated the principle of the method using a single anti-light-chain allotype affinity separation, we are investigating other means of producing similar mixed molecules in sufficient amounts for therapeutic application *in vivo*. We will then be able to evaluate the therapeutic potential of monovalent monoclonal antibodies for the removal of unwanted cells in bone marrow grafts and for serotherapy of tumour cells.

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Anti-idiotypic cytotoxic T cells in rats with graft-versus-host disease

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Transfer of parental strain T lymphocytes into F₁ hybrid rats differing with respect to gene products of the major histocompatibility complex (MHC) causes a graft-versus-host (GvH) reaction. This reaction results from recognition of host allogeneic MHC gene products by specific clones of donor T cells¹. When given systemically in sufficient numbers, these donor T cells cause a progressive, generally fatal wasting syndrome, an early feature of which includes extensive splenomegaly^{2,3}. A more local, non-fatal GvH reaction, marked by extensive enlargement of the draining lymph nodes, ensues when donor T cells are administered via the footpad^{4,5}. Here, we demonstrate that cells derived from the enlarged draining lymph nodes of A/B F₁ animals undergoing local GvH disease caused by donor A T cells contain a subpopulation of host-derived killer T-cell precursors which can be activated to lyse specific blast cells, derived from mixed lymphocyte culture (MLC), reactive to host MHC^b alloantigens. These 'anti-idiotypic' cytolytic T cells lyse A anti-MHC^b MLC blasts, and also, they lyse anti-MHC^b MLC blasts from MHC different, third party rat strains.

Three particularly interesting features of GvH reactions are (1) cells of host origin contribute predominantly to the lymphoid hyperplasia⁶; (2) normal F₁ hosts are far more resistant to lethal GvH disease than are lightly irradiated F₁ recipients or hosts selectively depleted of T cells⁷⁻¹⁰, and (3) F₁ animals that recover from GvH disease caused by small numbers of parental T cells are profoundly and specifically resistant to GvH reactions caused by subsequent inoculation of T cells from the same donor strain; they remain, nevertheless, fully vulnerable to GvH reactivity caused by T cells from the opposite parental strain^{11,12}. The immune response we describe here may also account for those phenomena.

F₁ anti-parental killer cells were generated as described in Fig. 1, and the results of four independent experiments are summarized in Table 1. The first and second experiments show that L/DA lymph node cells, primed *in vivo* with parental strain DA (RTI^a) T cells, and restimulated in culture with DA anti-L (anti-RTI¹) MLC blasts, detect idiotypic markers on the

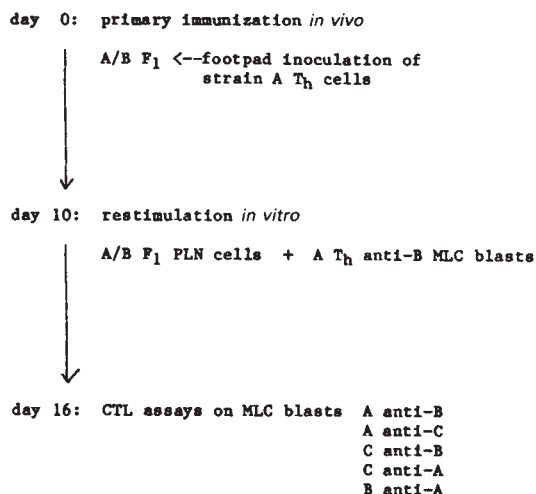


Fig. 1 Generation of F₁ T cells which lyse specific MLC blasts. F₁ rats were immunized in their hind footpads with 15×10^6 cells of the T helper subset of parental strain thoracic duct lymphocytes (TDL). This immunizing population was prepared by rosetting procedures¹⁷ using W3/25, a monoclonal antibody that identifies the T helper subset in rats¹⁴. Ten days later, dissociated cell suspensions were prepared from the 3-fold enlarged popliteal lymph nodes (≥ 150 mg) draining the site of the inoculation, and these (60×10^6) were stimulated in bulk cultures of W3/25⁺ parental TDL stimulated one or more times with host alloantigens. The media was supplemented (10% v/v) with supernatant from Lewis rat lymph node cells stimulated with concanavalin A ($5 \mu\text{g ml}^{-1}$; 5×10^6 cells ml^{-1}) for 48 h. After 5–6 days the surviving F₁ cells in the bulk cultures (10–20% yield) were tested for cytolytic activity in 4-h assays on a panel of ⁵¹Cr-labelled MLC blasts.

immunizing DA MLC blasts only if they have alloreactivity for L (RTI¹) MHC antigens. They lyse target MLC blasts of the DA anti-L (anti-RTI¹) combination, but not DA anti-BN (anti-RTIⁿ), nor MLC blasts of the other parent strain, L anti-DA (anti-RTI^a). They also show no lytic activity against a human erythroleukaemia line, K562, which is sensitive to lysis by rat natural killer cells.

The second, third and fourth experiments show that F₁ killer cells specific for idiotypes on parental strain blasts of the immunizing MLC combination are also lytic for MLC blasts from a third party strain having alloreactivity for the same host MHC haplotype. Thus in experiment 2, L/DA killer cells restimulated with DA anti-L MLC blasts lyse these target cells, and WF anti-L MLC blasts as well. The reverse is shown in the fourth experiment: L/DA killer cells, specific for L anti-DA

Table 1 Specificity of lysis by T cells obtained from rats undergoing GvH reactions

Expt	F ₁ recipient (RTI)	Cells for immunization		Target MLC blasts		MR/SR (c.p.m.)	% Specific lysis					
		1° <i>in vivo</i>	2° <i>in vitro</i>	Strain	(RTI)		Effector: target ratio	40	20	10	5	2.5
1	L/DA (l/a)	DA (a)	DA anti-L (a anti-l)	DA anti-l	(a anti-l)	2,747/855		42	45	38	36	20
				DA anti-BN	(a anti-n)	3,370/716		2	5	3	5	0
				L anti-DA	(l anti-a)	3,225/1,020		7	6	4	0	-1
				K562		10,293/669		0	0	0	0	0
2*	L/DA (l/a)	DA (a)	DA anti-L (a anti-l)	DA anti-L	(a anti-l)	1,884/506		46	43	35	30	
				DA anti-BN	(a anti-n)	3,317/698		8	9	7	6	
				WF anti-DA	(u anti-a)	1,912/459		0	0	3	0	
				WF anti-L	(u anti-l)	1,939/578		24	25	30	21	
				L anti-DA	(l anti-a)	3,496/799		5	4	5	6	
3	L/DA (l/a)	DA (a)	DA anti-L (a anti-l)	DA anti-L	(a anti-l)	2,336/1,155		50	53	42	31	35
				Aug anti-L	(c anti-l)	1,782/795		40	20	11	17	14
				Aug anti-DA	(c anti-a)	2,237/1,178		3	10	0	3	5
4	L/DA (l/a)	L (l)	L anti-DA (l anti-a)	L anti-DA	(l anti-a)	1,586/455		53	47	40	38	
				WF anti-DA	(u anti-a)	900/258		37	37	23	19	
				WF anti-l	(u anti-l)	1,006/379		-11	-12	-9	-13	
				DA anti-L	(a anti-l)	1,673/431		-6	-12	-7	-8	

* A slightly modified protocol was used for the preparation of killer cells in expt 2; see Table 2.

Table 2 Phenotype of L/DA anti-DA GvH lymph node cells stimulated in culture

Monoclonal antibody	Marker	% Positive cells in FACS Total	Blasts
I 1.69	RT1A ¹	90.97	94.2
OX 19	Pan-T	90.40	87.97
W3/25	T helper	22.60	24.97
OX 8	T killer/suppressor	90.55	90.89
*		3.13	3.0
†		3.02	3.1

The killer cell population used in this experiment is the same as that described in expt 2, Table 1; it was prepared with a protocol slightly modified from the others. Here, the L/DA popliteal lymph node (PLN) population was enriched for the T killer/suppressor subpopulation by rosette depletion¹⁷ with W3/25 and OX 12, monoclonal antibodies specific for the T helper subset¹⁴ and immunoglobulin bearing cells¹⁸. The remaining OX 8⁺, W3/25⁺, OX 12⁺ population was then co-cultured with irradiated (3,000R) L/DA lymph node cells, as a source of antigen presenting cells, and with the T helper subset (W3/25⁺) of irradiated DA anti-L MLC blasts, as a source of antigen, in a ratio of 1:1:1 (120×10⁶ total cells) for 5 days. 2×10⁶ cells were exposed to monoclonal phosphate-buffered saline antibodies for 60 min at 4 °C and washed three times in Dulbecco's phosphate-buffered saline without phenol red. They were then exposed for 60 min at 4 °C to sheep F(ab')₂ anti-mouse immunoglobulin labelled with fluorescein isothiocyanate (FITC) (Cappel Lab), washed three more times and then fixed in 1% paraformaldehyde. The % is based on counts of ~20,000 cells.

* No primary antibody with FITC-labelled anti-mouse immunoglobulin.

† No primary antibody and no secondary antibody.

but not DA anti-L, also lyse third party WF anti-DA, but not WF anti-L MLC blasts.

Table 2 presents evidence, based on analysis of cell surface phenotypes, that the killer cell present in the restimulated GvH lymph node culture is a T cell of F₁ host origin. For this experiment, aliquots of the surviving, cultured cell population shown in Table 1, expt 2, were exposed to a panel of mouse monoclonal antibodies: I 1.69, specific for class I MHC antigens of the Lewis rat strain (RT1A¹) but not DA (RT1A^a); OX 19, a rat pan-T-cell marker¹³; W3/25, the rat T helper subset marker¹⁴; and OX 8, the rat T killer/suppressor subset marker¹⁵. The percentage of cells staining with these monoclonal reagents was determined from fluorescence-activated cell sorter (FACS) profiles of all surviving cells, as well as the subpopulation of surviving blast cells gated by forward light scatter. At first glance, this experiment shows that F₁ cells of the killer/suppressor T-cell subset comprise the majority of cells (>90%) in the L/DA anti-DA GvH lymph node preparation after it was stimulated with irradiated DA anti-L MLC blasts in the presence of irradiated L/DA antigen presenting cells. Although DA blasts carrying Lewis antigens in their receptors might account for the binding of I 1.69, the finding that L anti-DA MLC blast targets are not lysed, whereas DA anti-L blasts are, makes this most unlikely (see Table 1, expt 2).

The finding that anti-idiotypic cytolytic T cells of host origin can be derived from the lymph nodes of F₁ animals undergoing sublethal GvH disease is of interest for three reasons. First, it seems likely that these cells reflect the existence of a cellular mechanism which renders normal F₁ animals much more resistant to lethal GvH disease than lightly irradiated animals⁷⁻¹⁰.

Second, these findings confirm and extend, with an *in vitro* system, an unexpected finding obtained in previous *in vivo* studies. We demonstrated that A/B F₁ rats immunized with small numbers of parental strain (A) T cells become profoundly and specifically resistant to GvH disease otherwise inducible with subsequent inoculations of T cells from the same donor strain¹¹⁻¹². This GvH resistance was shown to be mediated by host T cells having specificity directed towards a presumed 'specificity associated marker' present on parental A T cells having alloreactivity against host (MHC^b) alloantigens¹². Surprisingly, these markers (associated with anti-MHC^b alloreactivity)

present on the immunizing T-cell population from A donors also appeared to be present on T-cell subpopulations from other, third party, strains having alloreactivity for MHC^b. Anti-MHC^b GvH reactions, but not MHC^c GvH reactivity of these third party T cells were suppressed in A/B hosts immunized with A (anti-b) T cells¹⁶. Thus, the specificity of GvH resistance in these previous *in vivo* experiments parallels the lytic specificity of F₁ T cells against a conserved anti-MHC^b idotype seen in the present studies.

Finally, these results raise the interesting possibility that idiotypic structures or specificity associated markers of anti-MHC receptors specific for a particular MHC gene product, for example, MHC^b, are conserved on T cells in different strains and are more alike than on T cells from the same strain reactive to different MHC gene products. Moreover, it follows from this model that the cytolytic activity of F₁ T cells generated as described are apparently not restricted by MHC gene products as would be expected of CTL with lytic specificity for various (non-MHC) cell surface determinants. Alternatively, the F₁ CTL might be restricted by host antigen (MHC^b) occupying the receptor structures of MLC blasts, or have specificity for altered self MHC^b determinants created as a consequence of receptor binding. In either case, these findings may provide an interesting approach to analysis of the cellular events involved in the development of self tolerance and the down regulation of potential autoimmune responses to self MHC antigens.

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Transformation of NIH 3T3 cells by a human c-sis cDNA clone

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The mechanism of leukaemogenic transformation by human T-cell leukaemia/lymphoma virus (HTLV), a retrovirus implicated in the aetiology of certain adult T-cell leukaemias and lymphomas, is unknown but is conceivably associated with the expression of the cellular analogues of retroviral oncogenes. The HUT-102 cell line¹, derived from a cutaneous T-cell lymphoma and infected with HTLV²⁻⁴, expresses several cellular

oncogenes⁵. It is unusual among haemopoietic cell lines in that one of these is *c-sis*, the gene from which the oncogene *v-sis* of the simian sarcoma virus was derived, and perhaps the gene for platelet-derived growth factor (PDGF)^{6,7}. To explore the possible role of *c-sis* expression in HTLV-induced disease, we have obtained cDNA clones of *c-sis* from HUT-102 cells. Here we describe two such clones and report that one of them transforms NIH-3T3 cells. This is the first example of transformation of NIH-3T3 cells by a human *onc* gene other than *c-ras* or *Blym*, as well as the first demonstration of transformation by a human cDNA clone.

Polyadenylated RNA was prepared from the HTLV-producing HUT-102 T-cell line using the guanidine hydrochloride method⁸. A cDNA library was prepared from this RNA using the plasmid primer method⁹ with the plasmids pcDVI and pLI. The resultant clones contain a simian virus 40 (SV40) enhancer and early promoter, splice site, and polyadenylation signal, enabling the expression of the cloned message in eukary-

otic cells⁹. Using a cloned probe for *v-sis*¹⁰, subcloned into M13, two cDNA clones of *c-sis* were isolated (pSM-1 and pSM-2) from ~150,000 clones screened. In each instance, five clones surrounding the positive clones on the secondary plates were picked. DNA from these clones was negative with the *v-sis* probe.

One clone (pSM-1) contains a 2.7 kilobase (kb) insert and the other (pSM-2) a 2.6 kb insert. Restriction endonuclease digests show that each clone contains two *Bst*EII sites (Fig. 1A). Both *v-sis* and the corresponding region in a genomic λ clone¹¹ of *c-sis* have a single *Bst*EII site. In *c-sis*, this site is located in the last exon of the open reading frame (S.F.J. *et al.*, in preparation). When pSM-1 is digested with *Bst*EII plus *Cla*I, two fragments are produced of 1.2 kb and 0.8 kb which hybridize with the *v-sis* probe (not shown). Thus the 5' *Bst*EII site shown in Fig. 1B corresponds to the one contained in the last exon of the open reading frame of the *c-sis* gene. From this, we conclude that the *c-sis* RNA probably contains 1.9 kb of 3' untranslated sequences which includes the other *Bst*EII site, and that pSM-1 contains nearly 0.8 kb of coding sequences which extend approximately 200 basepairs (bp) upstream from the *v-sis*-related sequences. This confirms the presence of additional 5' coding regions in *c-sis*¹². A similar restriction endonuclease digestion pattern is obtained with pSM-2, except that the 5' portion of the gene is 50–100 bp shorter (Fig. 1A). A restriction endonuclease map of pSM-1, as well as the region which is homologous to *v-sis*, is shown in Fig. 1B. The restriction maps of the region of homology of both genes are identical.

Since expression of *c-sis* in T-cell lines is unusual⁵, we analysed HUT-102 DNA for evidence of rearrangement of *c-sis*. Using pSM-2 as a probe, we compared restriction endonuclease digests of DNA from HUT-102 and from an uninfected B-cell line (CR-B)¹³ from the same individual. The digestion fragment sizes are identical with both *Eco*RI and *Bam*HI (Fig. 2). DNA from the T-cell line 8402 was also tested using pSM-1 as a probe. When human cellular DNA is digested with *Eco*RI, *Bgl*II, and *Xho*I, pSM-1 detects the same bands that are detected by *v-sis*¹² as well as additional bands (not shown) which apparently hybridize to the additional *c-sis* coding sequences in this clone.

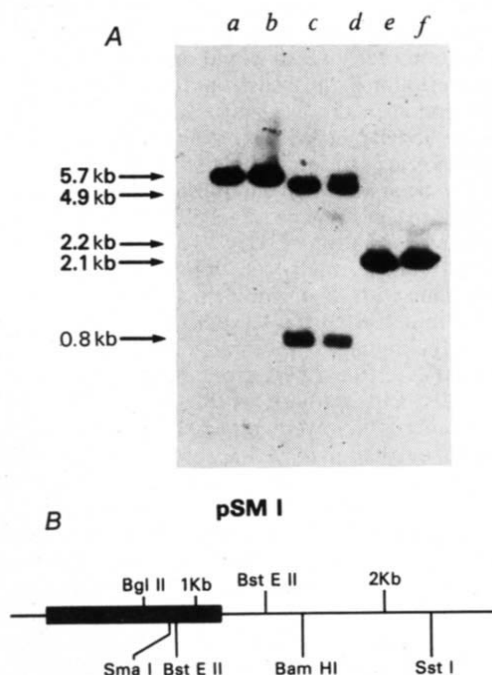


Fig. 1 pSM-1 and pSM-2. **A**, 200 ng of DNA from the plasmids pSM-1 (lanes b, d, f) and pSM-2 (lanes a, c, e) were digested with the restriction endonucleases *Cla*I (lanes a and b), *Bst*EII (lanes c and d) and *Bam*HI (lanes e and f). Arrows indicate the size of the detected fragments in kb, calculated from a standard curve using *Hind*III-digested λ phage DNA as a marker. **B**, Homology of *c-sis* with *v-sis*. A restriction endonuclease map of the plasmid pSM-1 cDNA insert is shown. The dark bar shows the area of homology with *v-sis*.

Methods: A cDNA library using HUT-102 mRNA was made essentially as described previously^{8,9} except that 11.5 μ l of repaired cDNA was used to transfect 500 μ l of competent χ 1776 cells. Ten aliquots of χ 1776 cells were transformed, mixed with an equal volume of χ -broth and incubated for 1 h. 750 μ l of cells were spread on a 137 mM nitrocellulose filter (Millipore, HATF) overlaid onto χ -broth agar plates with a 25 μ g ml⁻¹ ampicillin. 1.6×10^6 transformants per μ g of vector primer (pcDVI) were obtained. Transformed colonies were replica-plated, and screened as described previously¹⁸. 200 ng of DNA from pSM-1 and pSM-2 were digested with the indicated restriction endonuclease (2 units μ g⁻¹ DNA) in the buffer recommended by the supplier [*Cla*I (BRL, Gaithersburg) *Bst*EII (New England Biolabs) and *Bam*HI (Boehringer-Mannheim)] for 16 h at 37 °C. The DNA was then electrophoresed through 0.8% agarose, transferred to nitrocellulose, and hybridized to 1×10^6 cpm of ³²p nick translated probe. A subclone of *v-sis* described elsewhere¹² was cloned into M13. The cloned insert was purified by excision and gel electrophoresis from the double stranded M13 replicative form, and used for nick translation.

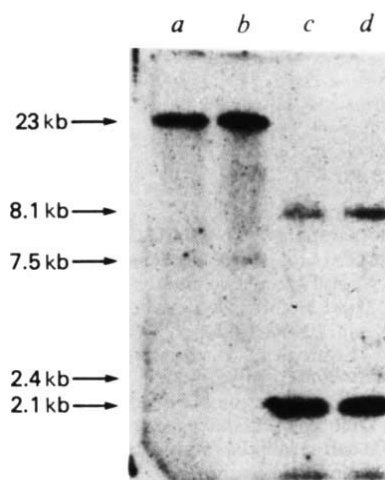


Fig. 2 Restriction endonuclease fragments of genomic DNA which label with pSM-1 and pSM-2. High MW DNA isolated from HUT-102 cells (lanes a and c), and CR-B cells (lanes b and d), was digested with *Eco*RI (lanes a, b) or *Bam*HI (lanes c, d) and hybridized to ³²P-pSM-2. Arrows indicate the size of the detected fragments in kb calculated from a standard curve using *Hind*III-digested λ phage DNA as a marker.

Methods: Digestion of DNA with restriction endonucleases were performed as described for Fig. 1. One μ g of plasmid DNA was nick translated with ³²p-dATP and ³²p-dCTP to a specific activity of 4×10^8 c.p.m. μ g⁻¹. 25 μ g of DNA was electrophoresed through 0.8% agarose, transferred to nitrocellulose, and hybridized to 3×10^6 c.p.m. of the indicated probe as described in Fig. 1.

To test whether NIH-3T3 cells could be transformed by pSM-1, cells were transfected with pSM-1 using the calcium-phosphate method¹⁴. After 18 days, the plates were examined for transformed foci of cells. Approximately 200 foci were observed per μg of pSM-1 DNA (Fig. 3A). These foci appear to differ morphologically from those which are typical, in our experience, of cells transformed by *ras*, in that the cells have a less spindle-shaped appearance while retaining the ability to

pile up into a typical focus. Several foci were picked using a cloning cylinder and the resulting transformed cell clones were expanded for DNA extraction. Southern blots confirmed the presence of pSM-1 DNA in the transformed cells (Fig. 3B). Digestions with *Bam*HI and *Cla*I indicated that the entire pSM-1 sequence was present in all the transfectants. Digestion with these enzymes and the no cut enzyme *Eco*RI showed that in all the transfectants, the pSM-1 was integrated at numerous sites. In many cases, however, the pSM-1 was also present at least transiently as an episome. Using the *v-sis* insert from M13 as a probe moderately high levels of *c-sis* RNA could be detected by Northern and RNA dot blots (not shown). Control NIH-3T3 cells transfected with calf thymus DNA resulted in no transformed foci.

There are several possible reasons for the transformation by pSM-1 of NIH-3T3 cells. First, the observed transformation may simply depend on a high intracellular concentration of PDGF, the putative product of the *c-sis* gene. Although externally added PDGF does not transform¹⁵, availability of PDGF receptors may limit the intracellular concentration which can be achieved. Furthermore, the pSM-1 construct utilizes the SV40 early promoter⁹, which would result in a high constitutive level of transcription in the transfected cells. Second, it is possible that an alteration in the *c-sis* gene in HUT-102 cells confers transforming activity on pSM-1. When high molecular weight HUT-102 DNA is used to transfect NIH-3T3 cells, no transformed foci are seen (E. W., unpublished observations), which suggests that the *c-sis* gene of HUT-102 is not intrinsically capable of transformation of NIH-3T3 cells. However, the *c-sis* promoter may not normally function in NIH-3T3 cells, or the intact *c-sis* gene with its promoter may be too large to remain functionally intact after transfection. Either possibility might also explain the finding that although many sarcomas express a 4.2 kb *sis* mRNA, the DNA from most does not transform NIH-3T3 cells, and of those which do, a relative of the *ras* oncogene family is involved rather than *c-sis*^{16,17}. Third, the cloned *c-sis* sequences in pSM-1 are truncated, lacking approximately 1.4 kb of 5' sequences of *c-sis* mRNA. The sequences that are present extend 150–200 bp beyond the 5' region homologous to *v-sis*. The extra sequences contain a methionine codon (ATG) 21 codons upstream from the sequences homologous to *v-sis* which is in phase with the *v-sis* open reading frame (S.F.J. *et al.*, in preparation). It is possible that the upstream *c-sis* sequences not contained in pSM-1 code for a regulatory region of the protein and that the absence of these sequences result in the observed transformation. Deletion of 5' sequences as a result of cloning may thus mimic the generation of the *v-sis* gene in SSV. We are currently attempting to obtain full-length clones of *c-sis* mRNA to answer these questions.

The data presented here unequivocally demonstrated that the *c-sis* message obtained from HUT-102 contains all the sequences necessary for transformation. This transformation potential could be relevant to transformation in some cases of HTLV-associated lymphoma, as well as in soft tissue sarcomas and glioblastomas, which also express *c-sis*¹⁵. It does not, however, seem a likely mechanism in all cases since expression of *c-sis* was not found in the majority of HTLV transformed cells.

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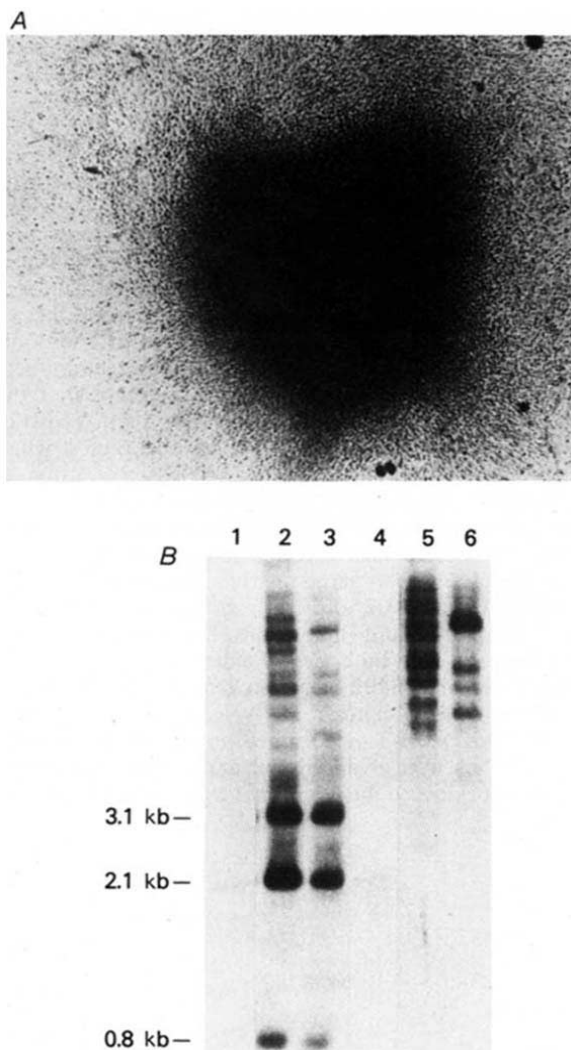


Fig. 3 A, Photograph ($\times 32$) of NIH-3T3 cells transformed by pSM-1. B, Southern blot of DNA obtained from the parent NIH-3T3 cells (lanes 1 and 4) and two different transformed cell clones, T98-7₃ (lanes 2 and 5) and T98-1₂ (lanes 3 and 6), digested with *Bam*HI (1–3) and *Eco*RI (4–6). ³²P nick translated pSM1 was used as probe. The arrows show the sizes of fragments generated by digestion with *Bam*HI of pSM-1 DNA.

Methods: NIH-3T3 cells were obtained from D. Lowry and were maintained on Dulbecco's modified Eagles medium with 10% heat inactivated fetal calf serum. For transfection, cells were seeded at 2.5×10^5 per 35 mm plate on the day before use. The transfection procedure used was a modification of that described by Graham and van de Eb¹⁴. Briefly, 20 μg of carrier calf thymus DNA was mixed with 0.5 μg of pSM-1 in 0.5 ml 250 mM CaCl_2 . This was then added dropwise with N_2 bubbling to 0.5 ml of $2 \times \text{HBS}$ solution at pH 7.0 and the calcium phosphate DNA precipitate was allowed to form over a period of 30 min at room temperature. 0.5 ml of this precipitate was then applied to each 35 mm plate containing 1.5 ml media and allowed to incubate at 37 °C for 4–6 h. The plates were then washed gently with fresh media once and re-fed and incubated an additional 12–16 h before splitting. After splitting, plates were held for 14–18 days and foci scored. Representative foci were then picked using 6 mm cloning cylinders and the resulting cell lines were grown up for further analysis. Southern blots were performed as described in Fig. 2.

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Determination of the leukaemogenicity of a murine retrovirus by sequences within the long terminal repeat

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Although the murine retrovirus SL3-3 is highly leukaemogenic^{1,2}, in both the structure of its genome and in its properties of replication in tissue culture it closely resembles the non-leukaemogenic retrovirus Akv (refs 3, 4). An earlier investigation of the properties of recombinant SL3-3-Akv viruses localized the major determinant of leukaemogenicity outside the *env* gene, in a region of the viral genome that includes the *gag* gene and the noncoding long terminal repeat (LTR)⁴. To localize the determinant of SL3-3's leukaemogenicity more precisely we have now constructed a recombinant provirus containing the LTR of SL3-3 and the coding region of Akv. The leukaemogenicity of these recombinants demonstrates that the determinant of leukaemogenicity lies within the SL3-3 LTR. Nucleotide sequencing of the LTRs of SL3-3 and Akv shows that they differ by a set of changes in the region thought to contain a transcriptional enhancer element. We suggest that enhancer region sequences are the major determinants of leukaemogenicity in these viruses.

The murine retrovirus SL3-3 was isolated from a cell line that had been established from a spontaneous T-cell lymphoma of an AKR mouse^{5,6}. Akv is an endogenous retrovirus that is expressed in AKR mice from mid-gestation onwards^{5,6}. Whereas SL3-3 induces lymphomas in several strains of mice, usually within 2–4 months post-inoculation, Akv is non-leukaemogenic^{2,7}.

To test whether the leukaemogenicity of SL3-3 is determined by its LTR, a recombinant viral genome, RECAS-LTR, was constructed between molecular clones of the SL3-3 and Akv genomes (Fig. 1). A 665 base pair (bp) segment of the AKR genome, beginning 36 nucleotides from the 5' end of the Akv LTR and extending 75 nucleotides into the untranslated leader

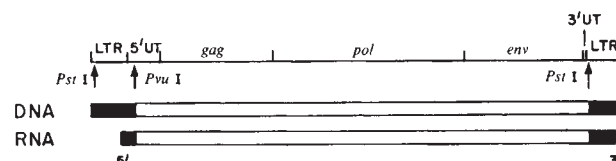


Fig. 1 Structure of the RECAS-LTR viral genome containing the SL3-3 LTR and the Akv coding sequences. Structures of the provirus and the corresponding RNA genome are shown. Sequences derived from SL3-3 are shown in black, and extend from the *Pst*I site located 36 nucleotides from the 5' end of the LTR to the *Pvu*I site located 75 nucleotides from the 5' untranslated leader sequence located downstream of the LTR⁴. Sequences derived from Akv are shown in white. Details of the general approach used to construct the plasmid containing the recombinant genome have been described previously⁴. Specifically, the *Pst*I–*Pvu*I fragment containing the SL3-3 LTR was subcloned into pBR322 to generate plasmid pLTR-S3. A restriction enzyme fragment that contains the SL3-3 LTR was isolated from plasmid pLTR-S3 and ligated to a restriction fragment from a clone of the Akv genome (pAKR-34) that contains the complementary fraction of the viral genome, lacking the LTR sequence. The resulting recombinant plasmid contains a viral genome that contains only a single LTR. This was used to transform *Escherichia coli* to tetracycline resistance, and the RECAS-LTR genome was cloned. Both LTRs of proviral genomes resulting from transfection of this plasmid are derived from the single SL3-3 LTR. The structure of RECAS-LTR plasmid was confirmed by digestion with the restriction enzymes *Bst*NI, *Kpn*I and *Bgl*II, which distinguish the recombinant structure from the parental plasmids⁴. The methods used to transfect the recombinant plasmid to yield infectious virus of the structure shown have also been described previously^{4,52}.

sequence, was replaced with the corresponding region from SL3-3. All the coding sequences of RECAS-LTR are derived from Akv.

Viral particles derived by transfection of NIH 3T3 fibroblasts with RECAS-LTR DNA were tested for leukaemogenicity by injection into newborn mice of the strains in which SL3-3 is leukaemogenic. As shown in Table 1, RECAS-LTR is leukaemogenic in the same strains as SL3-3, but the relatively low incidence of disease in inoculated NFS/N strain mice and the increased latent period in AKR/J and C3Hf/Bi mice shows that the leukaemogenicity of RECAS-LTR is actually slightly less than that of SL3-3.

LTR sequences of murine leukaemia viruses are not known to encode proteins, but do determine several viral functions^{8–12}. To investigate the precise nature of the differences within the region of SL3-3 substituted for Akv in the LTR recombinant virus, the nucleotide sequence of the SL3-3 LTR region was determined and compared with the corresponding sequences previously reported for Akv⁸ (Fig. 2). This comparison shows that the SL3-3 and Akv LTR sequences are of similar size and have the same general organization. The region that encodes transcriptional promoter¹³ and polyadenylation signals¹⁴ is identical. Upstream of the region that in both viruses is made up of

Table 1 Leukaemogenicity of RECAS-LTR

Virus	Leukaemogenicity* (no. with disease/no. inoculated—incidence—mean latent period (days))			
	AKR/J†	C3Hf/Bi	CBA/J	NFS/N
RECAS-LTR‡	24/28—86%—150	11/13—85%—140	1/2—50%—120	2/17—12%—99
Akv§	0/17—0%	0/9—0%	0/2—0%	0/13—0%

* Newborn mice, usually less than 48 h old, were injected intraperitoneally with 0.05–0.1 ml of virus stock. RECAS-LTR stocks contained approximately 10^5 infectious units per ml, as measured by end point dilution on NIH 3T3 fibroblasts. Mice were monitored daily. Diseased individuals were killed and necropsied to determine whether a lymphoma was present. All mice recorded as being diseased showed gross pathologies typical of thymic lymphomas, including greatly enlarged thymus, spleen, peripheral and mesenteric lymph nodes and liver.

† Leukaemogenicity in AKR mice was measured as a shortening of the latent period before the mice became moribund. Only mice that did so before 180 days of age were counted, so as to distinguish virally-induced from spontaneous disease. Leukaemogenicity in C3H, CBA and NFS mice was measured by induction as these strains normally do not have high spontaneous incidence of lymphomas.

‡ Two independent plasmid isolates of the RECAS-LTR genome were tested. As both isolates gave equivalent results on leukaemogenicity testing the data from them is pooled.

§ Pathogenicity data for Akv is pooled from several independent plasmid isolates, including the one used to construct the RECAS-LTR recombinant plasmids.

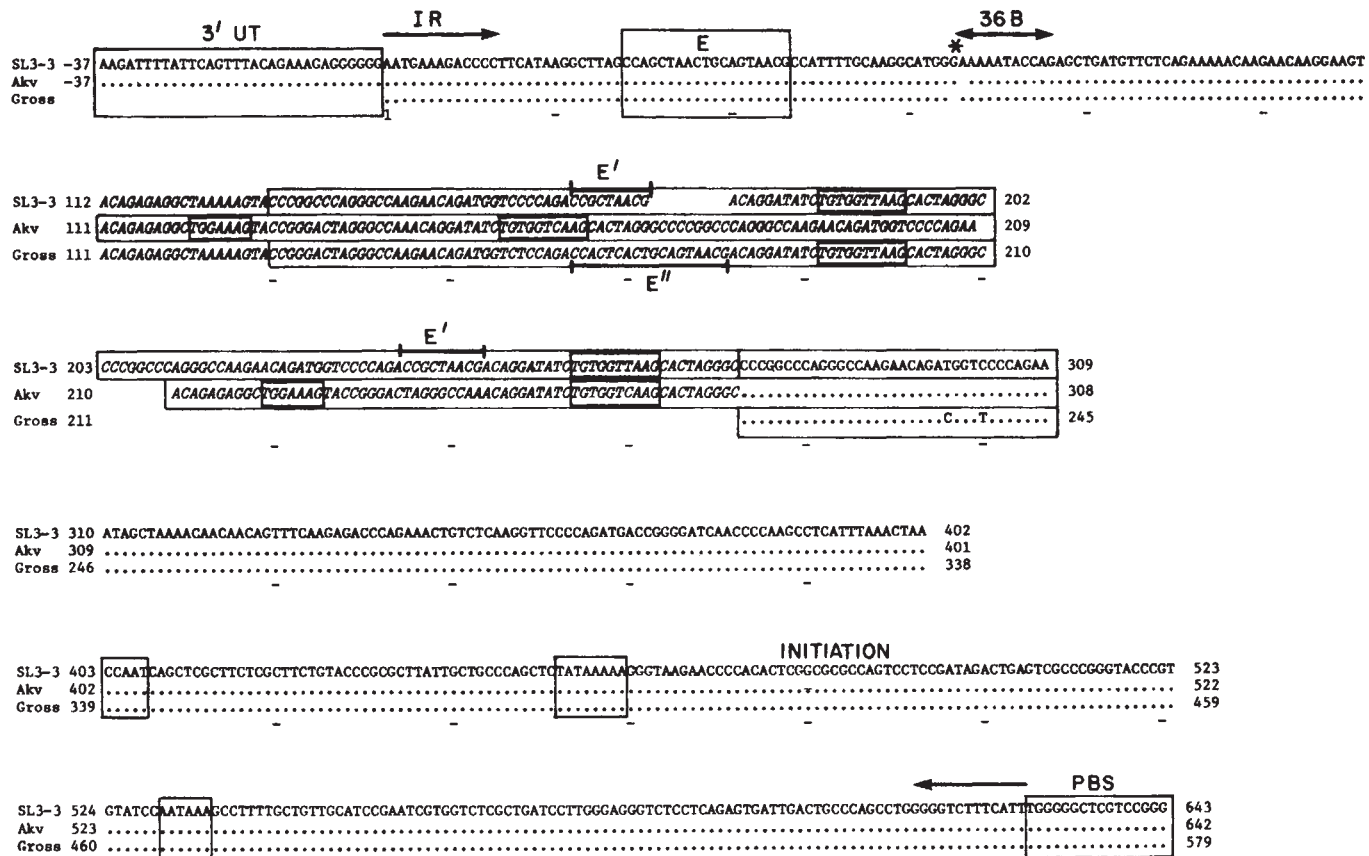


Fig. 2 Nucleotide sequence of the SL3-3 LTR region compared with the sequences of the Akv⁸ and Gross A¹⁹ LTR regions. Sequencing was performed using both the Maxam-Gilbert⁵³ and M13-dideoxy techniques^{54,55}. LTR sequences are shown along with the 3' untranslated sequences (3'UT) that flank one LTR in the complete viral genome, and the tRNA primer binding site (PBS) that flanks the other LTR. A dot indicates an equivalent nucleotide in the Akv or Gross A sequences relative to SL3-3. Sequences in italics comprise the direct repeat region of Akv, and the equivalent sequences of SL3-3 and Gross A. The large, thin-lined boxes show the boundaries of the direct repeat regions of each virus. Thick-lined, small boxes show the positions of sequences similar to the enhancer core sequences^{26,27}. The sequence designated E (see Fig. 3) partially matches the sequences labelled E' and E'' in the direct repeats of SL3-3 and Gross A. The positions of sequences of known significance that are located outside the direct repeats are also shown. The star indicates the position of the only nucleotide difference between the SL3-3, Gross A and Akv sequences that is located outside the direct repeats. The inverted repeats at the ends of the LTR are shown by arrows. Positions of the conserved sequences CCAAT, TATAAAAA and AAUAAA are boxed and the position where transcription initiates is also shown.

tandem direct repeats (see below) there is only one nucleotide difference between the two sequences: the insertion in SL3-3, relative to Akv, of an A residue 65 nucleotides from the 5' end of the LTR (Fig. 2). This alteration was previously noted as an oligonucleotide marker present in the genomes of several independently isolated, leukaemogenic viruses from AKR mice, including those of SL3-3 and Gross passage A virus (Gross A)^{2,15,16}. The sequence of the SL3-3 leader sequence downstream of the primer binding site is identical to the equivalent sequence of Akv (M. Etzerodt and F. Pedersen, personal communication) through the point where the recombinant virus was constructed.

In striking contrast to these conserved regions of the two LTR sequences, the tandem repeat sequences are markedly different. In the Akv LTR two copies of a 99 bp sequence are present as direct repeats (Fig. 2). The comparable region of SL3-3 consists of two 72 bp repeats, followed by a third copy of the first 35 bp of the repeats. Despite these differences there are similarities between the directly repeated sequences of the two viral LTRs. The SL3-3 repeat region can be seen to be related to that of the Akv LTR by two deletions, an insertion of a sequence similar to that of a segment upstream of the direct repeats, three single base changes and a tandem duplication. These changes are summarized schematically in Fig. 3.

These differences between the SL3-3 and Akv LTR sequences must account for the leukaemogenicity of the LTR recombinant virus. In this context, it is interesting to compare the LTR

sequence of another leukaemogenic virus, Gross A, with that of SL3-3. Gross A is the prototype leukaemogenic virus derived from AKR mice¹⁷. Recombination studies have shown that the major leukaemogenic determinant is localized within a region that includes the viral LTR, 3' untranslated region and the 3' half of the *env* gene¹⁸. This observation raises the possibility that the Gross A and SL3-3 LTRs contain similar leukaemogenic determinants.

Comparison of the LTR sequence reported for Gross A¹⁹ with that of SL3-3 shows that they are strikingly similar (Figs 2, 3). The LTR sequences of both viruses are identical to that of Akv outside of the direct repeats, except for a single nucleotide difference, which occurs at the same position in both SL3-3 and Gross A (Fig. 2). However, unlike the single nucleotide insertion in SL3-3, the difference in Gross A relative to Akv is a change of G to A.

Within the direct repeat region, the Gross A LTR is very similar to that of SL3-3, and correspondingly different from that of Akv (Fig. 3). Deletions relative to Akv show that the Gross A LTR has a general structure which is equivalent to that of SL3-3. An insertion in the repeat region relative to Akv occurs at the same position in Gross A as that in SL3-3. Although this insertion has a similar sequence to that of SL3-3 at the 3' end, it is nine nucleotides longer. Both inserts are similar to a sequence near the 5' end of the LTR (Figs 2, 3). There are six single base changes in the region of the Gross A LTR which is similar to sequences in the direct repeats of Akv (Fig. 3). Three

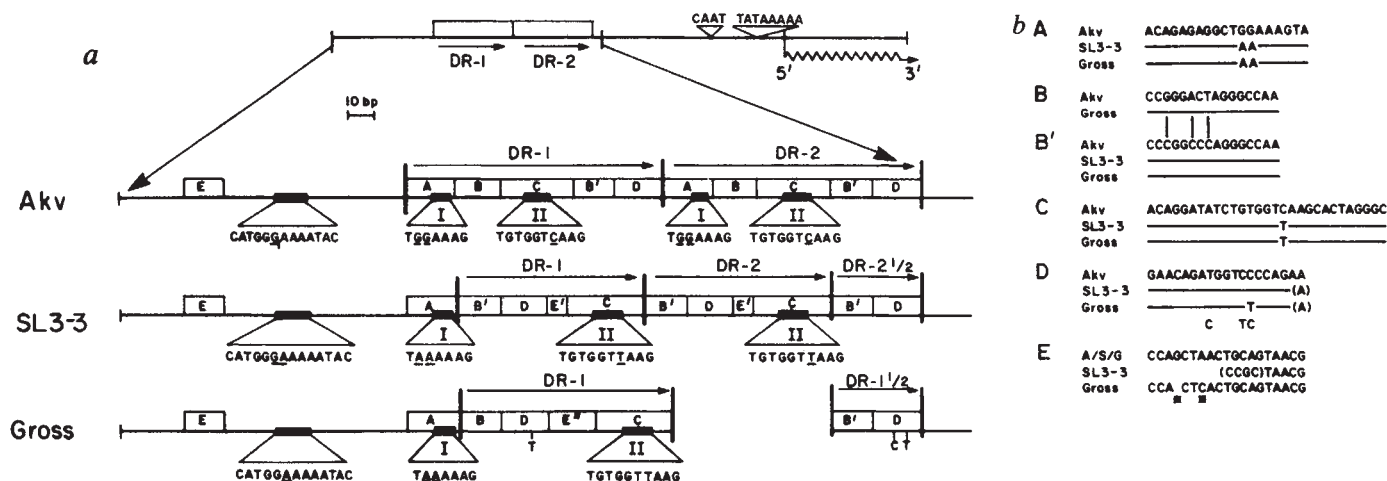


Fig. 3 Schematic representation of the relative configuration of sequences comprising the direct repeat regions of Akv, SL3-3 and Gross A. The general organization of the Akv LTR is shown at the top. The direct repeat region from the 5' end of the LTR to just beyond the 3' end of the direct repeats is expanded and shown below, along with the equivalent regions from SL3-3 and Gross A. Each direct repeat of Akv is subdivided into five segments, designated A, B, C, B' and D. The sequence of these segments in Akv is shown at the bottom. B and B' are identical at 13 of 16 nucleotides. The boundaries of each segment were determined by locating the equivalent segments in SL3-3 and Gross A. The sequences of these are also shown at the bottom, with a line indicating homology and the positions of differences being noted. SL3-3 can be defined as being related to Akv by two deletions, an insertion, three single base changes and a tandem duplication. Relative to the structure of the Akv direct repeats, these steps are deletion of segments B and C from the first Akv direct repeat and A and B from the second. An insertion designated E' is partially homologous to a sequence near the 5' end of the LTR labelled E, and may represent a duplication of it. Three single base changes are present in SL3-3 relative to Akv. These are shown in the sequences labelled I and II and are all located in the sequences that are related to the enhancer core sequence^{26,27}. The complete SL3-3 structure also requires a tandem duplication of 72 bp here shown as DR-2. Thus, this clone of SL3-3 consists of two complete tandem repeats DR-1 and DR-2, and 35 bp of a third repeat unit, DR-2½. The sequence reported by Villemur *et al.*¹⁹ for a clone of Gross A can be defined as being related to Akv by a similar series of steps. Deletion of segments C and B' and A and B followed by insertion of sequences E' generates a sequence equivalent to that of SL3-3. The three single base changes in sequences I and II of SL3-3 also occur in Gross A. Three additional single base changes are also found in the segments designated D. The overall structure of this clone of Gross A thus consists of 81 bp of a unit designated DR-1 and 35 bp of a second unit designated DR-1½. Note that only the 3'-most segments, labelled D in SL3-3 and Gross A, contain the deoxyadenosine residue that is shown in parentheses at the bottom of the figure. The sequence in the vicinity of the single nucleotide difference between the SL3-3, Gross A and Akv sequences that is located outside of the direct repeats is also shown. The similarity between the SL3-3 and Gross A LTRs is consistent with the hypothesis that this region of each virus is derived from the same locus, or family of loci, in the germ line of the AKR mouse¹⁶.

of these changes are identical to those observed in the SL3-3 sequence. In contrast to SL3-3, only a partial tandem repeat is present in Gross A. Nonetheless, the organization of the Gross A LTR is clearly very similar to that of SL3-3 (Fig. 3).

The observation that differences between the LTR sequences of the leukaemogenic viruses SL3-3 and Gross A and that of the non-leukaemogenic virus Akv are restricted to a region extending from the single nucleotide difference through the tandem repeats argues that the function associated with this region determines leukaemogenicity. The tandem repeat region of the viral LTR was identified as a transcriptional enhancer element in the murine retroviruses Moloney sarcoma virus (MSV)²⁰⁻²² and Harvey sarcoma virus^{23,24}. The direct repeat regions of Akv, SL3-3 and Gross A are clearly related to the corresponding region of MSV^{8,25} (Fig. 3) and, moreover, contain sequences similar to the 'core sequence' of identified enhancer elements^{26,27} (Fig. 2). It is therefore likely that this region functions as an enhancer in all these viruses.

We suggest that viral leukaemogenicity is determined by these putative enhancer region sequences. Specifically, we suggest that the enhancer of SL3-3 permits efficient transcription and replication of the virus during infection of a critical target tissue, whereas the activity of the Akv enhancer is deficient in such tissues. Likely targets are cells of the T lymphocyte lineage as the malignancy induced by SL3-3 is a T-cell lymphoma^{1,2}. Differential transcription and replication of the virus in other tissues, such as thymic epithelium, might also play a part in disease induction²⁸.

Several recent observations are consistent with the hypothesis that LTR sequences determine leukaemogenicity by conferring tropism for the pre-tumour cell. Viruses capable of inducing T-cell lymphomas replicate in the thymus, whereas non-leukaemogenic viruses do not^{29,30}, and LTR sequences of Gross

A virus have been shown to confer thymotropism³¹. Moreover, the erythroid or lymphoid nature of tumours induced by Friend leukaemia virus and Moloney leukaemia virus, respectively, is probably determined by the viral LTR sequences³². Recent evidence also indicates that tissue specificity is a property of both cellular and viral enhancer elements³³⁻³⁹.

It seems likely that the ultimate mechanism of tumorigenesis by murine leukaemia viruses involves the activation of specific cellular genes by insertion into the cellular genome of viral transcription regulatory elements⁴⁰⁻⁴⁵. Efficient viral transcription in the target tissue might be required to permit a high level of infection of the tissue, increasing the probability of the requisite integration event, and also for high levels of transcription of the cellular gene^{41,42,46}. Evidence from several investigators suggests that the viral *env* genes might also play a part in lymphomagenesis^{28,47-51}, and efficient transcription might also be required for high levels of expression of these genes.

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Non-function of a Moloney murine leukaemia virus regulatory sequence in F9 embryonal carcinoma cells

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Moloney murine leukaemia virus (M-MuLV) infection of embryonal carcinoma (EC) cells results in the integration of proviral DNA into the host cell genome¹⁻³, but not in virus production¹⁻⁶. One suggested explanation for the lack of viral gene expression in EC cells has been methylation of the integrated viral DNA¹. However, subsequent reports indicated that integration of the M-MuLV DNA occurs soon after infection, but that viral DNA methylation occurs considerably later^{2,3}. Nevertheless, viral gene expression is not observed even at early times. One possible explanation is that certain M-MuLV regulatory sequences do not function in EC cells. We now present evidence which supports this hypothesis.

Like M-MuLV, polyoma virus gene expression is restricted in EC cells⁷. Mutants of polyoma have been isolated that productively infect EC cells⁸⁻¹². The mutations that allow viral

Table 1 Infectivity of transfected proviral DNA clones in NIH 3T3 cells

3' LTR	Experiment		
	1	2	3
Mo			
DNA 1	2-10 × 10 ³	—	—
DNA 2	—	2.5 × 10 ³	10 × 10 ³
Δ Mo			
DNA 1	1 × 10 ¹	—	—
DNA 2	—	1.4 × 10 ¹	0.2-2 × 10 ¹
Δ Mo + PyF101			
DNA 1	3-10 × 10 ²	6 × 10 ²	10 × 10 ³
DNA 2	—	—	5.5 × 10 ²

NIH 3T3 cells were transfected with DNA from the different proviral clones described in Fig. 1 by the calcium phosphate method. The cells were transferred 1:5 1 day after transfection (before the spread of infectious virus), and scored for production of virus by the UV-XC cell assay¹⁷ upon reaching confluency (2-3 days). Transfections with 0.1, 0.01 and 0.001 μg of test DNA were performed, in the presence of 5 μg of carrier calf thymus DNA. Infectivities in XC plaque-forming units per μg transfecting DNA were calculated, and are shown in the table. Data are shown from three different transfection experiments, involving two different DNA preparations for each construct.

expression occur in the region of the polyoma genome identified to have transcriptional enhancing activity in transfection assays¹³. When restriction fragments encompassing these mutations are coupled to heterologous genes and used to transfect F9 EC cells, there is enhanced expression of the heterologous genes in comparison with constructions having no polyoma fragment, and also in comparison with those containing the analogous wild-type polyoma fragment¹⁴. The similar restriction of polyoma and M-MuLV in EC cells suggested that M-MuLV gene expression might also be blocked at the level of enhancer function.

We first tested whether removal of a specific regulatory sequence from the M-MuLV long-terminal repeat (LTR, Mo in Fig. 1) would affect the infectivity of M-MuLV in NIH-3T3 mouse fibroblasts. A recombinant subclone of M-MuLV DNA containing a deletion encompassing the tandemly repeated sequences located upstream (-341 to -182) of the promoter region (Δ Mo, Fig. 1a) was first isolated. The tandemly repeated sequences have previously been implicated as enhancer elements^{15,16}. This subclone was then used to construct a complete M-MuLV proviral DNA clone, containing a wild-type long terminal repeat (LTR) at the 5' end and the deleted LTR at the 3' end (Fig. 1b). This M-MuLV DNA was used to transfect NIH-3T3 cells, followed by assay for M-MuLV infectious centres using the XC syncytial assay¹⁷. The appearance of an infectious centre, revealed by a macroscopic XC plaque, is dependent on the spread of infectious virus from the initially transfected cell to neighbouring uninfected cells. Furthermore, during retroviral infection and DNA synthesis sequences present in the U3 regions of both LTRs originate from the 3' end of the viral RNA, which is itself encoded by the 3' LTR¹⁸. As a result, secondarily infected cells would contain deleted LTRs at both ends of the provirus. Table 1 illustrates the results of the transfections experiments. While transfection with wild-type M-MuLV proviral DNA (Mo) gave high levels of infectious centres, deletion of the tandem repeats (Δ Mo) greatly reduced the number. The low level of infectious centres resulting from constructions containing a Δ Mo 3' LTR probably resulted from recombination at the DNA level during transfection to give viral DNA with wild-type LTRs at both 5' and 3' ends (data not shown). It is therefore likely that removal of the tandem repeats from the M-MuLV LTR resulted in complete loss of infectivity.

We further tested if the loss of infectivity resulting from deletion of the M-MuLV LTR tandem repeats might be the result of deletion of sequences with transcriptional enhancer activity. An LTR was constructed in which the enhancer sequence of a polyoma virus mutant (PyF101) capable of productively infecting EC cells was inserted into the M-MuLV LTR

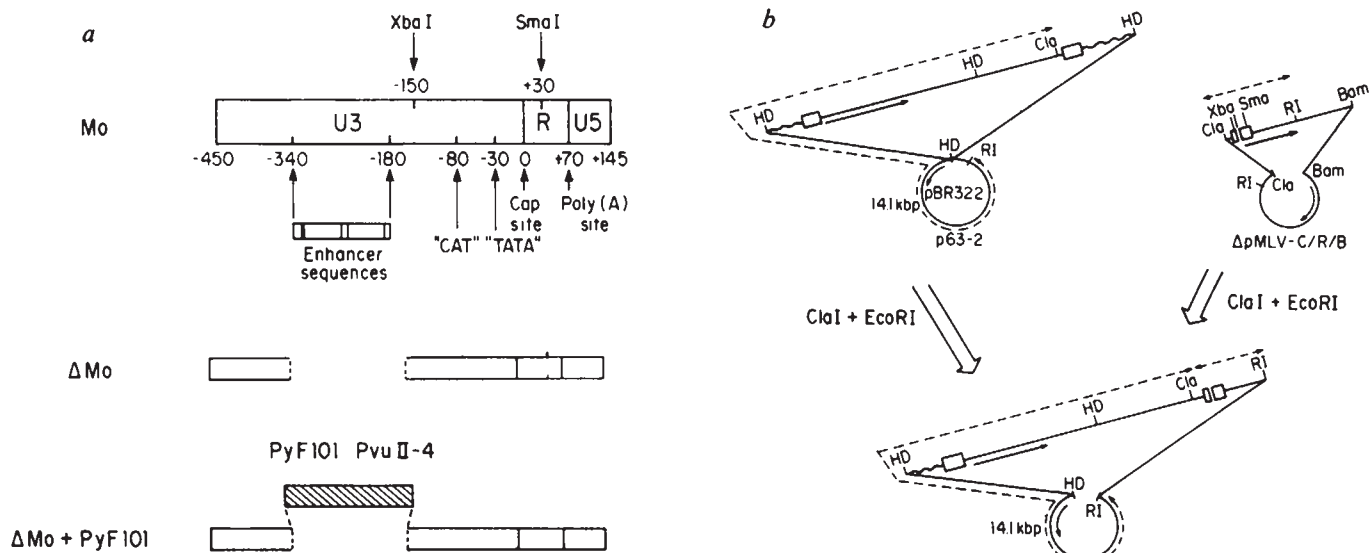


Fig. 1 *a*, LTRs used to construct complete proviruses for tests of infectivity and CAT plasmids to tests enhancer activity. Mo: this is an expanded view of the LTR from a clone of circularly permuted M-MuLV proviral DNA, pMLV-1a^{28,29}, and shows the sequences important in proviral transcription. U3 contains tandemly repeated sequences implicated as enhancer elements, and the consensus sequences CCAT and TATA. The initiation site and poly(A) site define the 5' and 3' ends of R, respectively; within R is the putative signal for polyadenylation AATAAA. The *Xba*I site used to construct the deletion Δ Mo, and the *Sma*I site used to link the M-MuLV promoter to the CAT gene are also shown. Δ Mo: this LTR contains a deletion extending from position -335 to -150 relative to the initiation site, which encompasses the tandem repeats. It was obtained by deletion about the *Xba*I site at -150 by exonuclease III and *S*₁ nuclease digestion, followed by re-ligation in the presence of *Xba*I linker. Sequences deleted to the 3' side of the *Xba*I site were restored by reintroducing a wild-type *Xba*I-*Sma*I fragment. Δ Mo+PyF101: this construct contains the *Pvu*II-4 fragment of mutant polyoma virus PyF101 within Δ Mo. Δ Mo+PyF101 was constructed by first adding *Xba*I linkers to the *Pvu*II-4 fragment, and then cloning into Δ Mo at the *Xba*I site. The polyoma fragment has the same orientation relative to the M-MuLV promoter sequences as it does with respect to the early region promoter of polyoma virus. *b*, The strategy used to construct complete M-MuLV DNA proviruses containing modifications in the 3' LTR. p63-2 is a pBR322 plasmid containing a complete proviral integration, subcloned from a recombinant bacteriophage λ clone isolated from M-MuLV-infected NIH-3T3 DNA³⁰. Δ pMLV-C/R/B is a pBR322 subclone of pMLV-1a from the *Cla*I site at the 3' end of the viral genome through the LTR to the *Bam*HI site in the 5' end of the genome, into which the deletion described in *a* was introduced. An additional modification was the conversion of a unique *Xho*I site in the 5' end of the viral genome into an *Eco*RI site. Ligation of the 14.1 kilobase (kb) *Eco*RI-*Cla*I fragment from p63-2 with the *Cla*I-*Eco*RI fragment containing the deleted LTR from Δ pMLV-C/R/B gave the final construct, containing a wild-type 5'LTR and a deleted 3' LTR. The same strategy was used to construct the provirus containing Δ Mo+PyF101 as the deleted 3' LTR. Wavy lines in the insert represent non-viral mouse cell DNA, and straight lines represent viral DNA. The uninterrupted and interrupted boxes represent wild-type and deleted LTRs respectively. Abbreviations for the following restriction sites: R, *Eco*RI; C, *Cla*I; S, *Sma*I; H, *Hind*III; X, *Xba*I; B, *Bam*HI. More detailed restriction maps for pMLV-1a and M-MuLV clone 63 are given in refs 29 and 30.

deleted of its tandem repeats (shown as Δ Mo+Py101 in Fig. 1a), and this was reconstructed to give a complete proviral organization. The inserted polyoma enhancer sequence was a 199 bp *Pvu*II fragment (fragment 4), obtained from the PyF101 F9 PyEC mutant¹⁰. This fragment has been shown to enhance transformation of EC TK(-) cells by the herpes virus thymidine kinase gene and to enhance expression of a bacterial chloramphenicol acetyltransferase (CAT) gene in *cis* during transfection of EC and NIH-3T3 cells¹⁴. Transfection of the reconstructed proviral DNA containing the polyoma PyF101 enhancers gave increased viral infectivity (Table 1) relative to the Δ Mo construct. Analysis of RNA from the transfected cells confirmed the presence of polyoma virus sequences (data not shown). These results indicated that the polyoma enhancer sequences can functionally replace the M-MuLV tandem repeats during M-MuLV infection, and support the possibility that the M-MuLV tandem repeats are enhancer sequences.

The transcriptional activities of the wild-type, deleted and substituted M-MuLV LTRs were also assessed in a transient expression system. For these experiments, portions of the LTRs were inserted into plasmids containing the bacterial CAT gene followed by the sequence AATAAA, which is thought to direct RNA polyadenylation (Fig. 2). The constructions included the entire promoter region of the LTR, so that transcription would begin at the LTR initiation site, read through the CAT gene and then terminate. The activity of the M-MuLV LTR promoter could then be determined by transfecting cells with the constructs and assaying for CAT activity¹⁹.

Three constructions were tested: Mo-CAT Δ Mo-CAT and Δ Mo+PyF101-CAT, which have the CAT gene linked to the

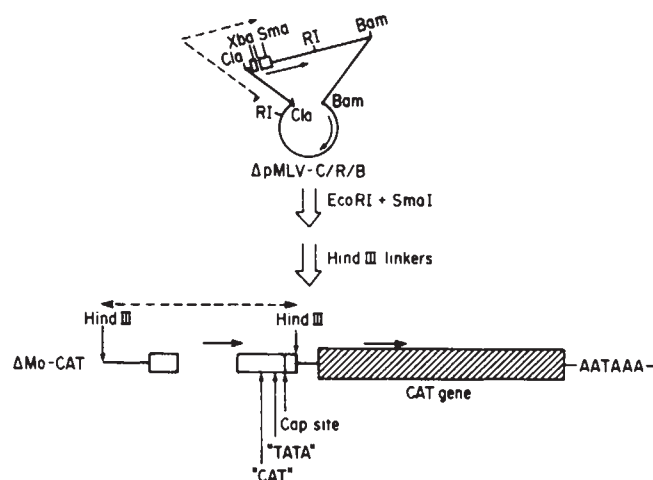


Fig. 2 The cloning strategy used to construct CAT plasmids containing the Mo, Δ Mo, or Δ Mo+PyF101 promoter regions. The construction of Δ Mo-CAT is shown, with the constructions of Mo-CAT and Δ Mo+PyF101-CAT proceeding similarly. Δ pMLV-C/R/B was digested with *Eco*RI and *Sma*I. The *Eco*RI site (in pBR322) and the *Sma*I site (in the R region of the LTR) of the U3-containing subfragment were modified to *Hind*III sites, and then cloned in plasmid pUCCATpA¹⁴ at the *Hind*III site 5' to the CAT gene. Clones containing the LTR fragment in the appropriate orientation to initiate transcription from the LTR promoter into the CAT gene were isolated. The sequence AATAAA represents an oligonucleotide which was directly cloned into the pUCCATpA plasmid 3' to the CAT gene¹⁴.

Mo, Δ Mo and Δ Mo+PyF101 LTRs respectively. Figure 3 shows results obtained from transfection of F9 EC and NIH-3T3 cells by these constructs.

CAT activity could be detected in NIH-3T3 cells transfected with Mo-CAT or Δ Mo+PyF101-CAT, indicating a functional LTR in both cases. NIH-3T3 cells transfected with Δ Mo-CAT showed no CAT activity, confirming the requirement of the tandem repeats for LTR promoter activity. In contrast, no CAT activity could be detected in F9 EC cells transfected with Mo-CAT, but CAT activity could be detected in F9 cells transfected with Δ Mo+PyF101-CAT. These data indicate that the M-MuLV tandem repeats do not function in F9 EC cells, and also

strongly suggest that the primary block in M-MuLV infection of EC cells is at the level of the tandem repeats, and possibly at the level of enhancer sequences.

Thus the upstream tandem repeats are required for the promoter activity of the M-MuLV LTR, and they can be substituted by polyoma sequences known to have enhancer activity. However, it is possible that the tandem repeats actually function within the LTR by a different mechanism from that of enhancer sequences. While we cannot completely rule this out, insertion of the polyoma enhancer sequences in the opposite orientations to those described here also restored activity to the Δ Mo LTR, suggesting that they are functioning as enhancers of transcription directed by the M-MuLV LTR promoter.

Several recent reports suggest that cell-specific activity of regulatory elements may be an important aspect of biological control. The enhancer elements associated with the mouse immunoglobulin heavy-chain gene appear to function only in lymphoid cells²⁰⁻²². The activity of viral enhancers from SV40, polyoma and murine sarcoma virus during transfection differs with the cell species^{16,23,24}. Tissue tropism and virulence of lymphoma caused by MuLV has been associated with the LTR, particularly the region containing the tandem repeats^{25,26}, and recently, cell-specific expression of the insulin and chymotrypsin genes was shown to be determined by the 5'-flanking regions²⁷. The results presented here may have revealed another example of a cell-specific regulatory element, the tandem repeats of the M-MuLV LTRs, which seem to promote transcription in fibroblasts, but not in EC cells.

Our results suggest that a primary block in M-MuLV infection of EC cells is the inability of the tandem repeats to function within the M-MuLV LTR. However, the viral infection cycle is a complex array of many events, and other blocks (including methylation) may also exist. The M-MuLVs containing PyF101 enhancer elements described in Table 1 provide tools for investigating this question, since they have overcome the primary block. Indeed, preliminary experiments indicate that infection of F9 EC cells with M-MuLV containing PyF101 enhancers results in expression of viral protein, while infection with wild-type M-MuLV does not.

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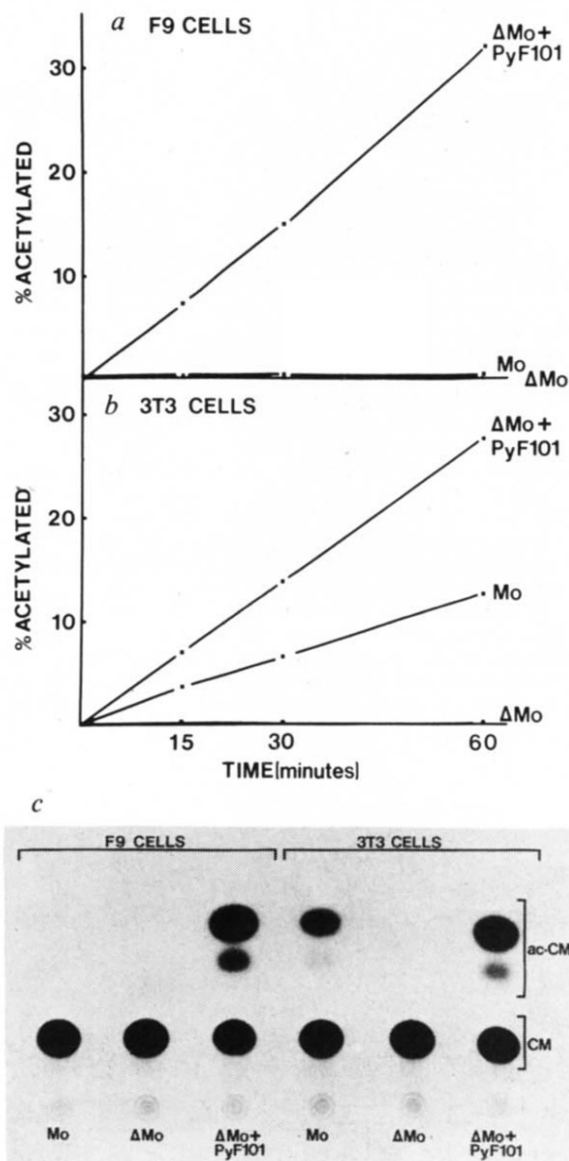


Fig. 3 CAT enzyme activity from transfected F9 EC cells and NIH-3T3 cells. Procedures of Gorman *et al.*¹⁹ were followed for the CAT enzyme assays. Transfections were performed as previously described¹⁴. **a**, Results of enzyme assays of F9 EC cells transfected with the three different CAT constructions described in Fig. 2. Acetylation of chloramphenicol is plotted as a function of assay incubation time. **b**, Results of enzyme assays of NIH-3T3 cells transfected with the three different CAT constructions. **c**, Autoradiograph of CAT enzyme assay from F9 EC cells and NIH-3T3 cells transfected with the recombinant DNA constructions Mo-CAT, Δ Mo-CAT and Δ Mo+PyF101-CAT. The autoradiograph was from the 60 min time point of the enzyme assays. These results are typical of repeated transfections and repeated enzyme reactions. ac-CM, acetylated ¹⁴C-chloramphenicol; CM, ¹⁴C-chloramphenicol.

Year of the eruption

Peter J. Smith

Krakatau 1883: The Volcanic Eruption and its Effects. A Centennial Retrospective.

By Tom Simkin and Richard S. Fiske.

Smithsonian Institution Press: 1983. Pp. 464. Hbk \$25; pbk \$15. (\$1.50 for postage.)

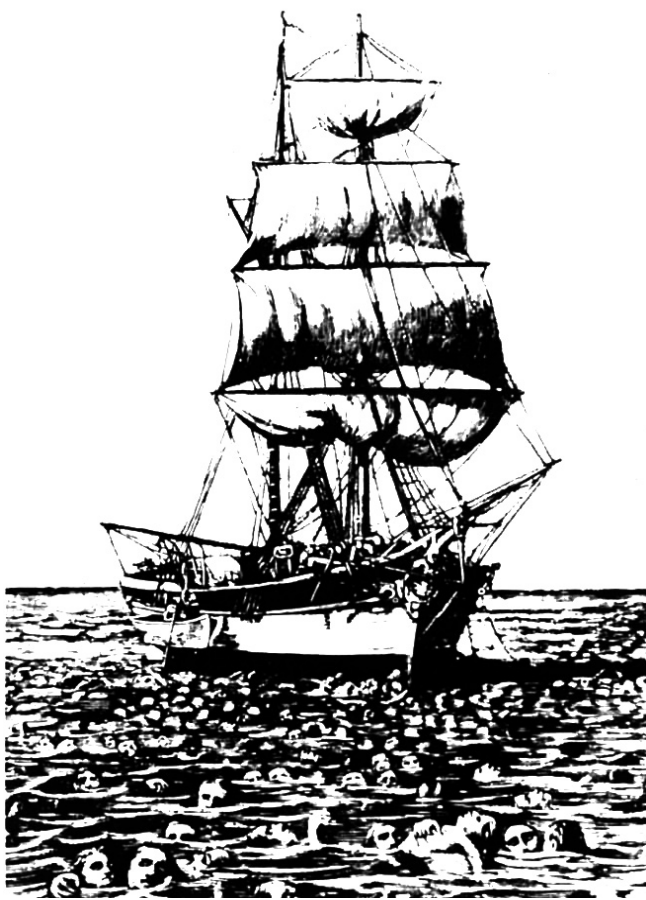
IT WAS the year of Brahms's Third Symphony, Renoir's *By The Seashore* and Richard Jefferies's *Story Of My Heart*. The Statue of Liberty was presented to the United States by France, Brooklyn Bridge opened in New York, the Boys' Brigade was founded in Glasgow, Sir Joseph Swan unveiled artificial silk and dynamite gangs were busy blowing up London. The previous year had witnessed the deaths of Charles Darwin, Ralph Waldo Emerson, Jesse James and Karl Marx. Stravinsky and Picasso were one year old, Bartok was two and Einstein was four.

It was also the year in which a telegram reached Singapore from Batavia, the capital of the Dutch East Indies, informing its recipients that "During night terrific detonations from Krakatau (volcanic island, Straits Sunda) audible as far as Soerakarta, — ashes falling as far as Cheribon — flashes plainly visible from here". As Simkin and Fiske point out in their magnificent centennial volume, it was a telegram whose importance to science lay as much in the medium as the message. The 1883 explosive eruption of Krakatau (or Krakatoa as the British mistakenly call it) is remembered ostensibly for its remarkable size and spectacular distant effects; but although it was large by modern standards, it was far from the most violent known. Its 18 km^3 of tephra was as almost nothing compared to the $1,000 \text{ km}^3$ blasted out by the eruption that formed Lake Toba (1,200 km away) about 75,000 years ago. Even the 1815 eruption of Tambora, 1,400 km away, had ejected about 100 km^3 of rock.

The real reason why Krakatau was to become, and remain, the most famous of all volcanic events is that it was the first eruption of any size to have had its worldwide effects extensively documented, a phenomenon made possible only by the existence of the 40-year-old electric telegraph. News of the eruption was telegraphed around the world within hours. Within days and weeks, the world's newspapers and magazines were able to report that 165 villages had been wiped out, that more than 36,000 people had died, that

40-metre tsunamis had swept 600-tonne blocks of coral before them, and that only a third of the original island still lay above sea level.

The significance of all this for science was that, for the first time, what would otherwise have appeared as disparate



A fanciful view of one of the consequences of the eruption of Krakatau, a ship passing through a sea choked with corpses (from *La Fin du Monde*, by Flammarion, 1894). Many corpses were in fact reported by ships in the area, but these were usually associated with blankets of floating pumice and tangles of vegetation.

events around the Earth could be causally related. Every paragraph in the world recorded the air-wave from Krakatau over a period of five days. Tide gauges reacted to the sea-wave thousands of miles away. Blue and green suns were observed for 13 days; and red sunsets were generated for up to three years. Ten months after the eruption, pumice rafts reached the opposite side of the Indian Ocean. Volcanic dust shielding out solar radiation lowered global temperatures for up to five years. In an age of instant satellite communi-

cation, it is difficult to envisage just how remarkable it must have seemed at the time to be able to perceive all such natural phenomena as stemming from a single event.

The rest is history, albeit history with a startling lacuna, as Simkin and Fiske reveal. The Royal Society's appeal in early 1884 for eyewitness and scientific accounts of the eruption and its aftermath soon resulted in an extensive archive from which the Society was able to compile its now-famous report of 1888. But despite all this effort, the key document — R.D.M. Verbeek's official report of 1885 — was never translated into English from either its original Dutch or French editions.

Verbeek, a mining engineer who had mapped the geology of Krakatau only three years before the eruption, was an ideal choice as chief investigator; but most of the resulting monograph, comprising a narrative, before-and-after field studies and relevant data from government sources, was to remain hidden from English-reading eyes.

In providing the first translation of a large part of Verbeek's text, Simkin and Fiske demonstrate that for the past century the extant version of the Krakatau story has been the volcanological equivalent of the princeless *Hamlet*. Moreover, much of the rest of the play has not been as complete, or at least as readily accessible, as it might have been, which is why they surround the Verbeekian core with 87 eyewitness accounts and more than 100 pages of scientific (geological, oceanographic, meteorological and biological) reports, mostly published since 1883, showing how understanding of the eruption and its consequences has progressed.

To these are added a 27-page chronology (to 1981), an extensive annotated bibliography, reproductions of Verbeek's 25 chromolithographs of scenes seven weeks after the eruption, many rare (and some previously unpublished) photographs of the eruption and its

effects, reproductions of William Ascroft's paintings of Krakatau's atmospheric effects as observed in Chelsea, colour photographs of modern volcano-atmospheric effects for comparison, and numerous linking commentaries. The whole adds up to the most extensive and authoritative account of Krakatau ever published; a more impressive centenary celebration could hardly be envisaged. □

Peter J. Smith is Reader in Earth Sciences at the Open University, and Editor of *Open Earth*.

Model exercise

Brian Charlesworth

Theory of Natural Selection and Population Growth.

By Lev R. Ginzburg.

Benjamin-Cummings/Addison-Wesley: 1983. Pp.160. \$21.95, £16.95.

THERE are two approaches to mathematical modelling in biology: one is to seek general formulations of the properties of basic processes, without paying much attention to observational detail; the other is to try and produce models that explain particular classes of facts. Both the approaches are clearly needed for proper theoretical understanding (although most biologists would probably sympathize more strongly with the second), and the great pioneers of population genetics each contributed in both of these ways.

Lev Ginzburg's book is an example of the first approach. Although he covers a number of largely unrelated topics, ranging from the dynamics of complex ecosystems to the population genetics of multi-allele and multi-locus systems, he is always concerned to extract generalizations about "macroparameters" from the jumble of underlying mechanisms. The book reads much more like Ginzburg's thoughts on a collection of topics than an attempt to

survey a field, and I feel that he could, in places, have made greater acknowledgement of the work of others — for instance in relation to the stability of randomly chosen fitness sets in multiple allelic systems (Chapter 4), and the interaction of selection and density-dependence (Chapter 6).

Ginzburg's approach is frequently original and elegant, as exemplified by the treatment of change in mean fitness in multi-locus systems (Chapter 3). Here he demonstrates that a randomly-chosen n -dimensional vector of gamete frequency is expected to be located near the centre of the space of allowable frequency values, and uses this to show that mean fitness will usually increase in an n -locus system.

Of course, the biologist will ask what one can do with such generalizations, and the answer is often unclear. In the n -locus case, for example, Ginzburg goes on to say that points in the neighbourhood of equilibria are not necessarily representative of randomly chosen points, so that his theorem does not rule out an important influence of recombination on the dynamics of change near equilibrium. My impression of this book is thus of a clever and enjoyable intellectual exercise but one which leaves me uncertain of its importance for evolutionary biology. □

Brian Charlesworth is Reader in the School of Biological Sciences at the University of Sussex.

More volcanology

ELSEVIER'S new monograph series, *Developments in Volcanology*, has got off to a flying start with the recent publication of three hefty tomes with similarly illustrated hardback bindings. Interior style is rather less uniform, however, two of the books (Vols 1 and 3) being printed via camera-ready typescript and the other having been typeset in the more traditional way.

Although all three are edited "symposium" volumes, the origins of the material also differ. The first, *Forecasting Volcanic Events* (edited by H. Tazieff and J.C. Sabroux; pp.635, Dfl. 185, \$78.75), comprises 37 specially commissioned chapters all of which are science-based but some of which also deal with the social, legal and economic aspects of volcanic hazards. Indeed, the book is intended not just for volcanologists but also for non-scientists, particularly those in local government and administration who are so often vexed by the inability of volcanologists to come forward with clear-cut predictions unhedged by provisos. Readers who persevere will certainly understand the nature of scientific uncertainty by the end.

The second book, *Arc Volcanism* (edited by S. Aramaki and I. Kushiro; pp.652, Dfl. 230, \$97.75), contains about 10% of the material presented at a 1981 conference

and is a straight reprint of Vol.18 of the *Journal of Volcanology and Geothermal Research*. The 25 papers, all of which are specialist-scientific, range widely over the geochemistry (mainly), geology and geophysics of island-arc volcanic activity. Specialists will know what they are in for and the rest of us will give up in despair.

Explosive Volcanism (edited by M.F. Sheridan and F. Barberi; pp.481, Dfl. 220, \$84.75), also reprinted from *J. Volcanol. geotherm. Res.*, comprises 20 papers resulting from a workshop held in 1982. Topics covered include the atmospheric hazards of volcanic activity, the past 5,000 years of activity at Mount Pelée, the little-known AD 472 eruption of Vesuvius, the fundamentals of hydrovolcanism, the eruption of ignimbrites, volcanism in the Aleutian arc, computer-simulation of ash transport and deposition, and the evolution of the Fossa cone of Vulcano, as well as more detailed studies of the Sacrofano-Baccano caldera in Rome and the Somma-Vesuvius complex. Diverse though the topics are, the subject of explosive volcanism as a whole is rather more accessible than that of the previous volume and the text is thus less forbidding. Indeed, because a number of the articles treat the subject from the point of view of volcanic hazard, the book will be of interest to the natural audience for the first volume.

Peter J. Smith

Cutting ecology down to size

John H. Lawton

The Ecological Implications of Body Size.

By Robert Henry Peters.

Cambridge University Press: 1983. Pp.329. £17.50, \$29.95.

How much does a rhinoceros eat? What would be a good estimate for the defaecation rate of a brontosaurus? What is the expected ratio of biomass for krill and whales in the Antarctic Ocean? Ecologists are often confronted by questions of this kind, but lacking the necessary data or the means of obtaining them have to guess the answers using reasonable assumptions. Undoubtedly "guestimates" are much improved if we know the sizes of the organisms involved, because there exists a rich set of relationships between the rate at which individual animals do things and their body size. These allometric relationships are the subject of Peters's book.

All biologists know of the existence of some allometric relationships, one of the most familiar being the "¾ power rule" for metabolic rates — that is, to a good approximation, individual metabolic rates for different species scale as the 0.75 power of their body weights, with three broad groups of animals (unicells, poikilotherms and homoiotherms) differing only in the intercept of the underlying relationship, not in its slope. But such relationships, most of them less well known, exist for many other animal attributes. By describing a large number of body-size relationships in standard form, Peters has performed an invaluable service both for ecologists and for physiologists. Sixty pages of appendices summarize hundreds of empirically determined equations linking body size to metabolic rates for various taxa under different conditions of temperature and activity, to physiological variables such as pulse rate or total sleep time, to costs of movement, to water and nutrient requirement, to home range size and so on. Here is a treasure house of information.

The bulk of the text is a lucid general account of the ecological utility of this information, commencing with conventional problems in what might broadly be termed physiological ecology. Good examples are the capacity of birds and mammals of different sizes to withstand low temperatures, and the contrasting costs of migration in animals that have to fly, swim or walk to their destination. This material occupies Chapters 3–6, following an introduction and a mathematical primer on such matters as logarithms and power functions in Chapter 2. The physiological theme resurfaces in Chapter 13 where Peters reviews explanations for why, in

power formulae in general, mass exponents of $\frac{3}{4}$, $\frac{1}{4}$ and $-\frac{1}{4}$ are so effective in describing biological phenomena.

Ecologists of a less physiological bent will probably find Chapters 7–12 the more interesting. For example, Chapter 7, "Ingestion", yields fascinating insights on prey mass as a function of predator mass, expected daily prey capture rates for predators of different size and so on, the predictions being obtained by combining "lower level" empirical relationships to yield some very sensible answers. Likewise, in Chapters 8 and 9 individual body-size relationships are used to predict patterns of population and community energy flow and nutrient cycling. For myself, however, Chapter 10 is the most intriguing. Here Peters abandons relationships derived from individual animals and looks at population phenomena, specifically at population density as a function of body size and at number of species as a function of body size. To a good approximation, average

population densities scale as the reciprocal of individual body mass over nearly 13 orders of magnitude of body size, whilst the number of species declines at somewhere between a -3 and -2 power of bodily length. Here are bold empirical patterns, crying out for verification, amplification and explanation.

Clearly this is an important book. It is different — no other ecology text deals so comprehensively with all these problems — and by ignoring cherished disciplines, by blending physiology and ecology, and by looking at problems with a fresh eye, Peters yields new insights and opens up new questions. It is also provocative, because it seeks patterns in nature where others have seen a mass of unrelated detail. More than anything else, it cuts many problems down to size. As Peters himself admits, size is not everything; but it certainly helps! □

John Lawton is a Reader in the Department of Biology at the University of York.

Science is people

G.H. Beaven

Chambers Biographical Encyclopaedia of Scientists.*

Edited by John Daintith *et al.*
Chambers/Facts on File: 1983. Pp. 599.
£35, \$80.

The Biographical Dictionary of Scientists. Vol. 1 Biologists; Vol. 2 Chemists.

General editor David Abbott.
Blond Educational: 1983. Vol. 1, pp. 182,
£10.95; Vol. 2, pp. 203, £10.95.

REVIEWING the *Biographical Companion to Modern Thought* in the *Times Literary Supplement* recently, the writer noted that "lives are somewhat more amenable to dictionary treatment than ideas". The comment is equally valid for brief scientific biographies, and the two works under review (subsequently referred to as Chambers and Blond) differ somewhat in their attempts to resolve the problem. In Chambers, the brevity of most of the 2,000-odd entries only allows the scientific achievements of a subject to be outlined in a sentence or two, though often with admirable clarity and conciseness. In Blond, with 200 or so names in each volume, more effort is made to explain the significance of the subject's discoveries in the context of the relevant discipline; for some reason this approach seems to be most successful in dealing with organic chemistry, where structural formulae (of a curiously old-fashioned and space-wasting style) are often included.

In both works, diagrams are sparsely used; they are frequently helpful, but sometimes trivial (for instance the Bunsen

burner). A more liberal use of illustration would have greatly helped the elucidation of scientific ideas attempted in Blond.

The two works differ in their appeal to the reader. Chambers encourages browsing, because some 6–10 names occur on each two-page spread. Opening at random (or even in search of a specific entry) invariably recalls a name, once familiar and now forgotten, last encountered as a biographical footnote in a long-discarded textbook. The style of the entries is somewhat varied, within the tight limits of brief biography, with some striking comments on parentage, which emphasizes the wide social backgrounds from which scientists emerged before careers in science became professionalized.

The personalities of the subjects also come through more consistently in Chambers, in which some of the entries (for instance on Alan Turing and Rosalind Franklin) are quite frank. Others are diverting: on Rumford, who married Lavoisier's widow and later suggested that Lavoisier was lucky to have been guillotined, or on Sir George Cayley, whose coachman was ordered, reluctantly but successfully, to flight-test the first man-carrying glider in 1853. Contemporary reactions to Herbert Spencer's views on

Social Darwinism are recalled in Darwin's remark that Spencer's habit was to think very much and observe very little, and in T.H. Huxley's devastating comment that Spencer's idea of a tragedy was a deduction killed by a fact. Blond is not so lavish on personal touches but does include a good comment on Onsager's lack of lecturing skills, causing his courses to be known as "Sadistical Mechanics" and "Advanced Norwegian".

There are some differences in the scope of the two works. Blond appears to give more space to recent or contemporary scientists, while historical figures from the Greek philosophers onwards are more frequent in Chambers. Completeness in works of this kind is hard to test. Of five famous names in the field of tropical diseases, Chambers includes four and Blond only one (Ross), though with a diagram of the life-cycle of the malarial parasite. Of five personalities in the field of stereochemistry in Blond, only Kenyon is missing from Chambers. And of 20 living figures working in molecular spectroscopy, many are found in Chambers, but few in the Blond *Chemistry* volume. C.R. Harington appears only in the entry for G. Barger in Chambers, and not at all in Blond; Pitt-Rivers is missing from both.

The overall coverage by Blond will however include four more volumes dealing with mathematicians, astronomers, physicists (no doubt including the missing spectroscopists), and engineers and inventors. When complete (in November of this year) Blond should provide some 1,200 entries at a probable cost of £66, to be compared with some 2,000 entries in Chambers for £35. But the two works are not directly comparable.

On balance both may be expected to be useful, though to different classes of readers. One can envisage Chambers being used by a textbook writer to put flesh on the arid bones of his subject. Blond should appeal more to younger readers wishing to follow the development of scientific concepts through the contributions of many scientists. For cross-referencing of this kind Blond includes an excellent index of related names and subjects. □

G.H. Beaven is a Senior Research Fellow in the Department of Biophysics, King's College, University of London.

Star trails above the 17-ft radio antenna at Dover Heights in Sydney, 1952. The picture is taken from *The Early Years of Radio Astronomy: Reflections Fifty Years after Jansky's Discovery*, to be published by Cambridge University Press next week (price £25, \$39.50). The book is edited by W.T. Sullivan III; among the 20 other contributors are G. Reber, B.Y. Mills, A.C.B. Lovell, V.L. Ginzburg and O. Gingerich.



*In the United States, *A Biographical Encyclopedia of Scientists*.

May teach you more of man

Fred S. Rosen

Immunology in Medicine, 2nd Edn.

Edited by E. J. Holborow and

W. G. Reeves.

Grune & Stratton: 1983. Pp.655. £55, \$88.

UNTIL recently, immunologists have in some way resembled a gaggle of mediaeval scholars bent on using established dogma to prove the existence of the deity. This scholasticism, which has been irksome to other scientists, has been relieved by the excitement generated by the applications of hybridoma technology and the great strides in understanding the complex genetics of the immunoglobulin genes, the major histocompatibility loci and the complement proteins.

The second edition of *Immunology in Medicine* contains very little information to guide readers through the labyrinth of these recent discoveries, even as they apply to clinical medicine. Yet this text is a very useful and compact primer for students and physicians who are seeking information about the immunological aspects of disease. Organ system by organ system, the contributors methodically cover practical information on immunological patho-

genesis, diagnosis and therapy of the whole range of immunologically related diseases. The book contains beautiful, instructive electron micrographs of mast cell degranulation and renal deposits of immune complexes and immunofluorescence of skin in immunological diseases. The text is well organized with informative tables and illustrations.

The many contributors are all well-known specialists and practical men who have achieved a uniform succinctness in their presentations — or who have been well disciplined by the editors. The reader will have to resort to the recently published *Clinical Aspects of Immunology* by P.J. Lachmann and D.K. Peters or *Clinical Immunology* by C. Parker if they seek more detailed information, for this volume is not pallid or emasculate with tortured scholarship but rather like Wordsworth's impulse from a vernal wood. □

Fred S. Rosen is James L. Gamble Professor of Pediatrics at Harvard Medical School.

Setting time aright

A. Rupert Hall

Gregorian Reform of the Calendar.

Edited by G.V. Coyne, M.A. Hoskin and O. Pedersen.

Specola Vaticana, I-00120 Città del Vaticano, Europe: 1983. Pp.321. \$20.

ON 24 February 1582 Pope Gregory XIII (1502–1585) signed the Apostolic Letter, or Bull, *Inter gravissimas*, imposing a new calendar. That calendar, the Gregorian, came into force in October in countries under the Papal jurisdiction, and was adopted in Britain in 1752, in Japan in 1873, in Bolshevik Russia in 1918, in China in 1929. The event of four centuries before was celebrated in August 1982 by a meeting of historians of astronomy at the Vatican Observatory, from which this book stems.

It is well known that calendrical problems arise from the incommensurable periods of revolution of the Sun and Moon, that the timing of the recurrence of Easter is a combination of religious definition with astronomical computation, and that the Gregorian reform was made possible by possession of a more accurate measure of the length of the tropical year than had been available to Julius Caesar. But who could recite the invaluable calendrical studies of the Venerable Bede and Robert Grosseteste, here studied by O. Pedersen and J.D. North? Who is aware that the Gregorian leap-year rule can equally well be derived from the Alfonsine Tables and the *De Revolutionibus* of Copernicus? That the true author of the calendar was Luigi Giglio, who died in 1576 (G. Moyer) or that Christoph Clavius, the leading Jesuit astronomer who was mathematical head of the calendrical

commission, described Copernicus as “the illustrious restorer of astronomy in this our own century, whom all posterity will celebrate and admire as another Ptolemy” (U. Baldini)?

For all who are interested in calendrical computations and their evolution this is an essential and also fascinating volume, from the able early-background article by Pedersen to the note on the Universal Calendar by F. Russo, which records the Vatican's contemporary opposition to any post-Gregorian reform which would require the intercalation of extraordinary days outside the weekly succession of Sundays.

The problems of the calendar often illustrate the truth that the best may be the enemy of the good, as when John Dee (in 1582) advised that the Gregorian discontinuity should be put at eleven days, rather than ten. The reasons for the delay in achieving a reform when the faults and their remedies were so long understood in principle were the constant pressure for more important measures of reformation in the late-mediaeval Church, and the tendency of experts to haggle over pointless details. Religious sentiments about the earliest possible date for Easter and the need to avoid synchronism with the Jewish Passover were less seriously obstructive. The Gregorian calendrical commission did of course have to decide arbitrary matters such as the date to be assigned to the vernal equinox, and the placing of “the new and full moons too late rather than too early because it would be a less error to celebrate Easter in the second month after the equinox than in the last month before the equinox” (A. Ziggelaar). The role of Copernicus is still somewhat enigmatic; he was certainly never called to Rome to give his opinion, as Galileo had it; he probably gave confidence to the reformers in the accuracy of their sums, rather than in providing new numbers.

Opposition to the Gregorian reform was based on such non-scientific arguments as the Pope's lack of authority to regulate the calendar for Protestants, the date of the equinox, and (from Michael Maestlin, Kepler's teacher) the impropriety of seeking a precise calendar when the Second Coming was hourly expected (H.M. Nobis). Tycho Brahe, Kepler and the majority of reasonable astronomers accepted the Gregorian reform as the best that was practicable (M.A. Hoskin).

Inevitably in a composite work of this kind devoted to a single historical event, there are numerous repetitions of material; not all of the papers are of equally wide interest, nor are all based on deep research. Half-a-dozen of them, however, are splendid pieces of work with their technical content plainly presented, and these will surely have a long life. □

A. Rupert Hall is historical adviser to the Wellcome Trust and Emeritus Professor of the History of Science and Technology in the University of London.

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New for experimental biology

The following pages feature new products from manufacturers exhibiting at the meeting of the Federation of American Societies for Experimental Biology (FASEB), held this year in St Louis, from 1 April to 6 April.

● The restriction enzymes *ApyI* (C⁺CA_TGG) and *DpnI* (G⁺ATC) are now available from Boehringer Mannheim. Both require methylated DNA as a substrate. *ApyI* can be used in conjunction with *Bst*NI and *Eco*RII, and *DpnI* with *Sau*3AI and *Mbo*I for studying methylation patterns in DNA.

Circle No. 106 on Reader Service Card.

● Amino acid analysis by HPLC is possible with a new Spherogel ion-exchange column, post-column reactor and fluorescence detector from Beckman Instruments Inc. The Model 230 post column reactor is optimized for ophthaldehyde (OPA) derivatization of primary amines and can also accept other post-column chemistries. Low picomole detection of fluorescent compounds is made possible with the Model 157 fluorescence detector. These new components can be incorporated into any manufacturer's HPLC system and may be ordered separately or purchased together.

Circle No. 107 on Reader Service Card.

● The Hoefer Mighty Small vertical electrophoresis unit is designed to give fast results in a small format. This hand-sized unit is 10 cm high and 11 cm wide, and casts a gel 8.3 × 7.3 mm in 0.5, 0.75, and 1.5 mm thicknesses. The unit draws heat away from the gel and distributes it throughout the buffer by sealing the gel sandwich against the vertical upper buffer chamber so that the inside plate of the sandwich functions also as a wall of the buffer chamber. The plate which separates buffer and gel is made of aluminium oxide, a substance 30 times more heat conducting than an equivalent glass plate, ensuring that gels do not overheat.

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● The Monoclonal Table (right) is a full-colour poster of selected Becton Dickinson monoclonal antibodies for clinical research in cellular immunology. It provides a convenient reference illustrating the primary reactivity of commonly used antibodies to antigens on human T cells, T cell subsets, LGL/NK cells, B cells, and monocytes in normal peripheral blood. A free copy of the table is available from the Becton Dickinson Monoclonal Center.

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These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.

● A new membrane system developed by Bio-Molecular Dynamics eliminates the adverse effects (adsorption, denaturation, concentration polarization, shearing, the need for constant attention, and dryness) associated with horizontal membrane systems. The vertical membrane configuration of the Micro-ProDiCon and Micro-ConFilt will yield an average 90% recovery (biological, molecular, enzymatic) in concentrations up to 250 mg per ml. An added feature is the choice of MWCO membranes and predetermined final volumes.

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● Two new electrolyte controls have been introduced for the Orion ion selective electrode-based Na/K analyser, Model 1020. Electrol-N (normal) and Electrol-P (abnormal) are serum protein-based liquid controls that are used without reconstitution or other preparation. Together Electrol-N and Electrol-P provide controls over the whole range of physiological electrolyte values. Unopened, refrigerated vials are stable for two years. Open, refrigerated vials may be used for seven days.

Circle No. 111 on Reader Service Card.

● A new method for the assay of 1 α , 25-dihydroxy vitamin D has been developed by Amersham International for endocrinology research. The system dispenses with the HPLC step in sample purification, making use of an extremely specific, high affinity binding reagent. Non-specific lipid interference is eliminated.

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● GPC+ is a firmware-based program for calculating analytical results in gel permeation chromatography (size exclusion chromatography). The GPC+ program, available from Spectra-Physics resides on an 8K PROM chip which plugs into the Spectra-Physics SP4200 computing integrator. The program provides a custom dialog for modifying the integrator for molecular weight distribution calculations and permits the calculation (and plotting) of calibration curves.

Circle No. 113 on Reader Service Card.

● Diamond Electro-Tech's polarographic equipment is described in a brochure covering the Chemical MicroSensor, the oxygen uptake chamber, the calibration cell and a selection of mini and microelectrodes. These systems are suitable for polarographic measurement of O₂ and H₂ in cells, tissues or gases.

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● New from Boehringer Mannheim, the Boehringer gene expression kit allows immunological detection of specific gene expression in microorganisms, using HRPO-coupled antibodies and visualization by a colour reaction. The kit offers a rapid and sensitive non-radioactive method for the screening of a maximum of 20 plates per day, with a minimum detection limit of 10–100 pg of protein antigen. The individual components of the kit provide sufficient material for some 50 tests and constitute peroxidase, bovine γ -globulin, HRPO substrate, all necessary buffers and 55 filter disks and nylon nets.

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The Monoclonal Table

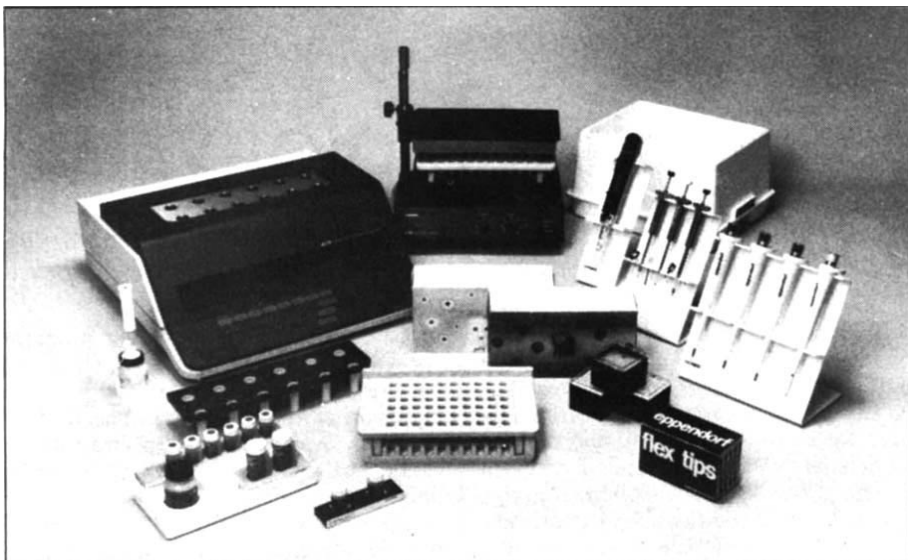
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● The FMC-Marine Colloids Division 1984 catalogue includes gel media systems and supplies for electrophoresis, isoelectric focusing cell culture and cloning. The catalogue also includes details of a new silver stain procedure for agarose gels. The catalogue lists product and technical procedures and applications literature.

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● "Magic", standing for "magnetic immunochemistry", is the trade mark for a new range of radioimmunoassay test systems. Manufactured by Corning, the tests are intended to offer the precision of centrifugation with the convenience of non-centrifugation. The key to the new Corning system is in the solid phase, where the antibodies are covalently bound to microscopic iron particles with high flotation characteristics. Unlike previous magnetic immunoassays, no mixing or shaking is required, and fast reaction kinetics shorten incubation times. Separation is by "side-pull" — two magnets located in the separation unit pull and tightly pack the particles onto the sides of the tube. Once separation is complete, which requires less time than it takes to load a centrifuge, the magnetic force continues to hold the particles in place during decanting. Magic RIA kits are now available for TSH, FT4, T4, T3 and T3U, with kits for FT3, B12/folate and digoxin available shortly.

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● Recent editions of United Technologies Packard application reports are available to gas chromatographers on request. Included are: a report showing the advantage of packed/packed columns, multi-dimensional chromatography and a specific detector for qualitative analysis of minor components in tobacco smoke condensate (item 07.95.230); a description of the analysis of organophosphorus insecticides using the on-column injector and flame ionization detector (item 07.95.231); an analysis of organosulphur compounds showing the flame ionization detector selectivity at the p.p.m.-level in hydrocarbon mixtures (item 07.95.232); and a demonstration of two of Packard's dedicated systems for the analysis of natural gas samples (item 07.95.233).

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● The Skatron, Inc. catalogue of immunoassay equipment includes Microplate EIA washers, a cell harvester for thymidine incorporation studies (MLC or receptor binding) and a supernatant collection system for chromium release cytotoxicity. Also covered are dispensers and other laboratory equipment.

Circle No. 120 on Reader Service Card.

● National Diagnostics' new histological catalogue describes a full line of chemical and solutions for the preparation of tissue slides for microscopic examination. Products mentioned include Histo-Clear, a clearing solvent which eliminates the hazards of xylene; and Mirsky's fixative concentrate, a fixing agent that contains no formaldehyde.

Circle No. 121 on Reader Service Card.

● Intended for permanent implantation in rats, Columbus Instruments cardiac output computers is Teflon coated and can be implanted for the same length of time as similar Teflon devices or catheters which are placed in arterial vessels. Microprobes are available in two diameters: 1/2mm and 1/3mm. Probe length is 76mm, and longer probes can be made on request.

Circle No. 122 on Reader Service Card.

● The Du Pont Prep I Automated sample processor is intended to replace many liquid-liquid extractions and manual column separations. The system combines centrifugation and column chromatography. Most extractions and manual column separations. The system combines centrifugation and column chromatography. Most extractions take 30 minutes or less, and present the user with concentrated or dried sample extracts. The unit includes a control and program selection panel, a parameter display, solvent storage, a centrifuge with unique bidirectional rotor, and disposable sample cartridges. Prep I is compatible with Du Pont 8800 liquid chromatography instruments.

Circle No. 123 on Reader Service Card.

● The new digital video time-lapse recorder from Micro Consultants is capable of capturing pictures of very slow movement or growth, and playing them back as a continuous sequence at near real-time rates. This unit is an option to Micro Consultants Intellect 100 PDS image processing system, and can be interfaced to either one or two 84 Mbyte Winchester disks. The recorder can operate at variable speeds with single frames being captured at pre-programmed rates or selected manually. Capture rates vary from near real time to one picture every 5 min. Areas of application for the time-phase recorder include the study of cell growth. Single frames of video can be selected and the images analysed on the Intellect 100.

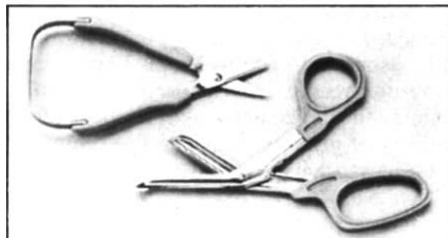
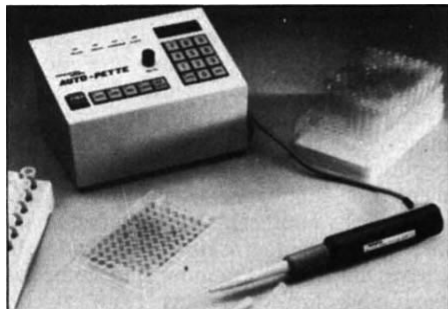
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● A new X-ray imaging system, the Nicolet Mikrox 3, is designed to look at small samples in fine detail. The system offers an X-ray source small enough that magnification by projection is practical with ability to image detail as small as 10µm. The system offers both photographic and real-time imaging. Images are displayed on a 13-inch monitor, magnified up to 100 times. Using the standard colour synthesizer, a colour image can be presented where differences in colour are related to small differences in X-ray density of the sample. This is especially useful where contrast is inherently low.

Circle No. 125 on Reader Service Card.



The Micro Consultants digital video time-lapse recorder in use with the Intellect 100 image processor



Wheaton's new automatic pipette, stainless steel shears from Bel-Art and the Harvard dual-range isometric transducer

● Rapid, highly specific sample quantitation by densitometry is possible with Schleicher & Schuell's Minifold II slot-blot system. This 72-place vacuum manifold is particularly useful for quantitatively screening large numbers of nucleic acid or protein samples. Samples are immobilized in a slot configuration of three rows of 24 slots, each with a filtration area of 6.0mm². Resulting autoradiograms or strained nitrocellulose can be easily scanned by standard densitometers. Quantitative results can be visualized rapidly due to sample concentration in a small surface area.

Circle No. 126 on Reader Service Card.

● A new addition to the Bel-Art Products range is a pair of heavy duty laboratory shears (model H37951). These stainless steel shears have large polypropylene handles which provide good leverage. The lower blade has a serrated edge to avoid slippage, and the foot on the front of the blade raises the shears above the work surface during travel. The shears cut a variety of materials including rubber and plastic tubing as well as light weight sheet metal. A notched handle is ideal for cutting wires. The shears are approximately 8 inches long with a 1½ inch cut and steam autoclavable at 250° F (121°C).

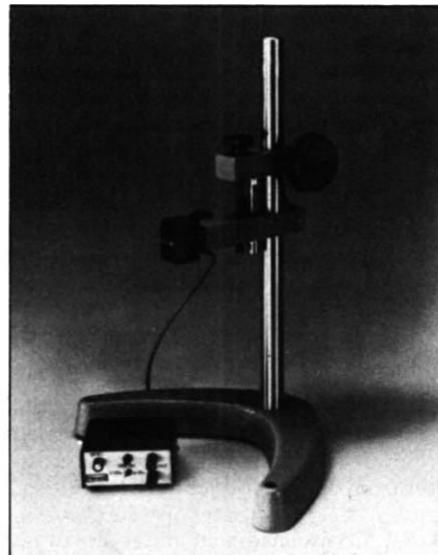
Circle No. 127 on Reader Service Card.

● A new CO₂/O₂ incubator from Tekmar/Heraeus makes use of a continuous flow sealed gas system which means that the consumption of both gases is minimized. The incubator senses the carbon dioxide with a thermal conductivity detector which adds gas only when it is needed, and oxygen is monitored with a Clark electrode.

Circle No. 128 on Reader Service Card.

● Harvard Apparatus's new dual range isometric transducer is complete with integral power supply and switch selectable 50 and 500 gram ranges. The transducer delivers up to 2.0 V direct current for use with any existing recorder. Beam deflection is only 0.1µm per gram.

Circle No. 129 on Reader Service Card.



● Ortho Diagnostic Systems produces a catalogue of the extensive range of Ortho-Mune monoclonal antibodies for identifying cells of the immune system. Included in the listing are several new products: OKB 2 and OKB7 for investigating B cell differentiation; OKM 5 for isolating and identifying monocyte subsets and platelets; Ortho B-cell sIg marker and Ortho FITC conjugated goat Anti-mouse IgG.

Circle No. 130 on Reader Service Card.

● The new Bio-Dot 96 well microfiltration apparatus performs rapid immunoassay, nucleic acid hybridizations and other filter assay procedures with no cross contamination between wells. Using the Bio-Dot apparatus, samples of unfractionated or purified protein, DNA or RNA are rapidly immobilized to chemically modified membranes such as nitrocellulose by using a vacuum force to pull the liquid samples through the membrane. Once bound, samples can be quantitated, hybridized or rapidly assayed for biological activities or determinants. Bio-Dot 96 is produced by Bio-Rad.

Circle No. 131 on Reader Service Card.

● The Wheaton Auto-Pette automatic pipettor comes with microprocessor control and remote pipettor unit(s), allowing the clinical technician to choose the optimum dispense mode to satisfy various pipetting parameters. Depending on the mode selected, the Auto-Pette will either function manually, automatically, or will cycle continuously. An automatic tip eject function switch allows the option of having the Auto-Pette automatically eject the disposable polypropylene tip after each dispense, or the tip may be ejected manually.

Circle No. 132 on Reader Service Card.

● The Shimadzu MPS-2000 is a UV/visible microprocessor-controlled recording spectrophotometer featuring a new dual-face end-on detector system and a saturation free pre-amplifier. The dual face detector system allows analyses of transmitted, scattered, reflected or refracted light.

Circle No. 133 on Reader Service Card.

● Lab Safety Supply Co. of Janesville, Wisconsin, has published a chart of value to anyone who handles chemical waste and must comply with the US Resource Conservation and Recovery Act. The 17×22 inch chart aids in identifying the classes of chemicals that are considered hazardous, and provides the actual hazardous waste number for over 650 chemicals. This chart helps to speed up paperwork procedures required under RCRA.

Circle No. 134 on Reader Service Card.

● Full details of the GIBCO range of products for the hybridoma laboratory are available on request. Products include fetal bovine serum, culture media, supplements, buffers and NUNC plastic labware.

Circle No. 135 on Reader Service Card.

● Sartopure pre-filters, from Sartorius, are entirely fabricated from polypropylene, and are available in pore sizes from 0.2 to 50 µm. Existing users of pre-filters can easily retro-fit Sartopure in any standard type of housing.

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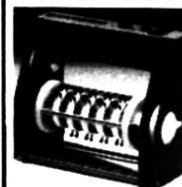
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● A free 8-page booklet on laminar flow (clean air) biological safety cabinets is published by Baker Company, Inc., a manufacturer of all types of laminar flow equipment. The publication discusses all types of cabinets available today, listing and describing them in terms of the NSF49 standard. It gives guidance on procedures for risk assessment based on NIH, NCI, CDC, and others.

Circle No. 137 on Reader Service Card.

● The Tekmar sonic disruptor is suitable for ultrasonic cell and tissue disruptions and operates over four power ranges from 250 to 2,000 W. Three of these ranges can energize two horns simultaneously, cutting processing time in half. Accessories include a wide range of standard horns and micro-tips and a special multi-element horn which will accept up to four microtips.

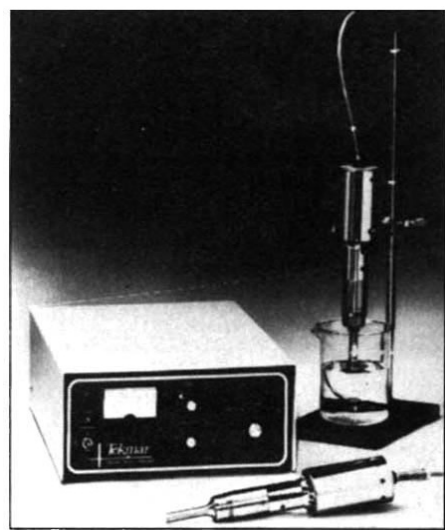
Circle No. 138 on Reader Service Card.

● The Bakerbond MAb column offers HPLC purification of monoclonal antibodies at speed with high sample recovery and purity. Using an MAb column, an IgG monoclonal antibody can be purified from mouse ascites fluid (centrifuged and dialysed) in less than 30 min with recovery greater than 90%. The isolated monoclonal antibody is also free of transferrin contamination. All sub-classes of the IgG immunoglobulin can be purified on Bakerbond MAb analytical and preparative columns, manufactured by J.T. Baker Research Products.

Circle No. 139 on Reader Service Card.

● The two major subpopulations of T lymphocytes in peripheral blood can be identified and enumerated without the use of a fluorescence microscope, using Bio-Rad's new Quantigen T4/T8 simultaneous assay. In the procedure, lymphocytes isolated from whole blood are incubated with antibody coupling microbeads in order to label T4 and T8 antigen bearing cells. The use of solid phase immunobeads eliminates the necessity of multiple washes required for second antibody techniques and obviates the need for a fluorescence microscope or flow cytometer.

Circle No. 140 on Reader Service Card.



Tekmar's 250-2,000 W sonic disruptor

● DEAE-Sepharose Fast Flow and CM-Sepharose Fast Flow are new ion-exchange gels giving rapid processing and throughput to the large-scale user. The gels, from Pharmacia Fine Chemicals, are derived from DEAE- and CM-Sepharose CL-6B gels by a modification of the cross-linking to improve mechanical strength. Flow rates of 3-5 times those of the CL-6B gel are observed. Also from Pharmacia, for DNA sequencing and isoelectric focusing, the ECPS 3000/150 is a high voltage (3,000 V) power supply now featuring a low range switch for control of power and current and new recessed output connectors for greater operator safety. Additional features include constant voltage, current or power regulation and automatic cross-over from one mode of control to another.

Circle No. 141 on Reader Service Card.

● Affinity-isolated goat antibodies to rat IgG ($\gamma + L$ chains) are now available from Tago, Inc. These antibodies are extensively affinity-purified in order to minimize cross-reactivity with human and mouse immunoglobulins. Goat anti-rat IgG is ideal as the second antibody with any rat monoclonal immunoglobulin of this class in indirect quantitative immunoassays and histochemical staining. Tago's anti-rat IgG is available unconjugated or tagged with fluorescein isothiocyanate (FITC) or horseradish peroxidase.

Circle No. 142 on Reader Service Card.

● Immunosoftware, from Dynatech, is an immunoassay (ELISA) software package system designed for use with Dynatech microplate readers MR 580, MR 600 and the MicroFluor system. As well as ELISA testing the system is applicable to a wide range of other colorimetric assays. In the standard processing modes, Immunosoftware defines up to 40 different procedures and templates routinely used by laboratories. These standard operations, once defined, are stored for future use. The non-standard processing mode is used to create templates and procedures and to set programme and process controls. In addition, the user can vary parameters, templates, processing and display routines at will.

Circle No. 143 on Reader Service Card.

● Easy labelling of sample tubes is provided by a new line of disposable 12 x 75 mm polystyrene tubes from Sarstedt, Inc., printed in colours and with graduations and an extra large writing block. Available with or without caps, the tubes are easily marked using pencil, felt tip or ball point pen and make it unnecessary to transfer samples to other containers for storage or subsequent processing.

Circle No. 144 on Reader Service Card.

● The 1984 catalogue from Vector Laboratories, Inc features more than 30 newly introduced products. The sections describing Vector's lectins, Biotin/Avidin System reagents, and Vectastain ABC kits are expanded to include specific information on the purification, properties and uses of each product.

Circle No. 145 on Reader Service Card.

● Two of the latest additions to the New England Nuclear range of research enzymes are endoglycosidase F (Endo F) and Endo H. Endo F is useful in establishing the molecular weights of the protein moiety of glycosylated proteins and peptides, and in establishing the involvement of sugars in the recognition of one molecule by another. Isolated from *Flavobacterium meningosepticum*, Endo F cleaves both high-mannose and complex asparagine linked glycans from glycoproteins, leaving the polypeptide backbone attached to a single sugar residue through the asparagine. Endo H, isolated from *Streptomyces plicatus*, useful in determining the molecular weight of the protein moiety of glycoproteins and their number of oligosaccharide chains.

Circle No. 146 on Reader Service Card.

● A new photometric microplate reader, the Dynatech M600, is suitable for ELISA and many other assays. A two-way computer connection and a wide selection of software packages make the instrument suitable for obtaining direct readings in the following applications: bacterial MICs, bacterial identification, CFT, blood group serology, monoclonal antibody studies, clinical chemistry, kinetic studies, protein and glucose determination.

Circle No. 147 on Reader Service Card.

● Millipore's Millex-FG filter unit is suitable for venting closed containers in pharmaceutical processing applications as well as non-aqueous solvent filtration up to 10 litres. The Millex-FG₅₀ unit incorporates a 0.2 μ m PTFE membrane in a bi-directionally-supported polypropylene housing, and provides 19.6 cm² of usable filter area in a compact, 58mm housing. The unit can withstand autoclave temperatures up to 130°C.

Circle No. 148 on Reader Service Card.

● The TANDEM-E TSH immunometric assay from Hybritech is a monoclonal antibody-based non-isotopic assay for the evaluation of thyroid function. The use of monoclonal antibodies in a solid phase two-site immunoenzymetric assay (IEMA) offers linear calibration, and the kit has a one year shelf life. TANDEM-E TSH is an enzymatic assay, so results are easily read at 405nm on a standard spectrophotometer.

Circle No. 149 on Reader Service Card.

● Becton Dickinson Monoclonal Center, Inc. now produces a one-step research test for determining the ratio of human T helper cells to T suppressor/cytotoxic cells in peripheral blood. These T cell subsets are simultaneously detected following treatment of the sample with monoclonal antibodies conjugated with two different fluorescent dyes. The antibodies in the test consist of a phycoerythrin (PE) conjugate of Anti-Leu-3a for T helper cells and a fluorescein (FITC) conjugate of Anti-Leu-2a for T suppressor/cytotoxic cells in a single reagent. The two antibodies are detected simultaneously under a standard fluorescence microscope.

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